MICROBIOTA AND METABOLOMIC CHANGES ACROSS VARIOUS CANINE GASTROINTESTINAL DISEASES

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2017

Major Subject: Biomedical Sciences

ABSTRACT

The canine gastrointestinal tract (GIT) is home to a complex ecosystem known as the microbiome. The microbiota (i.e., bacteria, viruses, fungi, and protozoa) are metabolically active, augmenting host digestion and thereby influencing the host, depending on both community composition and metabolic function. The upper GIT is physiologically different from the lower GIT, but feces are still frequently used as a representative sample due to the ease and non-invasiveness of collection. Previous studies in dogs as well as in humans have shown metabolic and microbiota changes in feces associated with various disease states. Our study aims were to characterize the microbiome at different sites of the canine gastrointestinal tract using sequencing and metabolomics; identify potential biomarkers in feces of dogs with chronic enteropathy (CE) by comparing their fecal composition with that in healthy dogs using sequencing and metabolomics; develop and analytically validate a quantitative assay for potential biomarkers; and use this assay to investigate altered concentrations associated with various gastrointestinal diseases in dogs at a single time point and over time.

Characterization of the contents of the duodenum, ileum, colon, and rectum revealed shifting compositions of both the microbiota and the metabolome. Major microbiota differences included distally increasing Firmicutes and decreasing Proteobacteria, while the differences in the metabolome largely reflected absorptive processes (e.g., decreasing concentrations of amino acids as these are absorbed by the host). In feces of dogs with CE, relative amounts of phytosterols were decreased and abundances of some fatty acids were altered. Thus a quantitative assay for fecal sterols and fatty acids ("FASter") was developed, analytically validated, and used to analyze samples from dogs diagnosed with CE, acute hemorrhagic diarrhea syndrome (AHDS), or exocrine pancreatic insufficiency (EPI). FASter profiles differed among the different groups of

dogs. The FASter profile of dogs with AHDS rapidly shifted towards that of healthy dogs, while in CE the profile exhibited little change over time.

Additional studies are warranted to continue to elucidate the role of lipid metabolism in canine gastrointestinal diseases, and determine whether fecal metabolite profiles can be of diagnostic use or even lead to a novel therapeutic approach.

DEDICATION

This manuscript is dedicated to the quadrupeds who inspired me to follow my path in veterinary research: Wilbur, Semi, Vanna Grey, Clever Elsie, Buddy, Fonn, Promenade, C.B., Lucy, Curry, Uma, Friday, Josephine Rogue, and Gitmeow. Your pawprints and hoofprints are indelibly left on my heart.

"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world." –Louis Pasteur

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Suchodolski, for his mentorship, encouragement, and unending optimism. I also thank my committee co-chair, Dr. Steiner, and my committee members, Dr. Alaniz, Dr. Ruaux, and Dr. Walzem, for their guidance and support throughout the course of this research.

My tenure at the GI Lab was filled with amazing scientific opportunities, and I owe a debt of gratitude to my friends and colleagues in the GI Lab for their part in making these possible and enjoyable. I would particularly like to thank Dr. Jonathan Lidbury and Nancy Cangelose, as well as everyone in the service lab. Quality research is only possible with excellent support; you all work hard to keep everything and everyone on track. My fellow graduate students – just, thank you. All of you have been amazing, especially those who talked me through when all I could see were obstacles to success.

I would not have had samples without the dogs and their owners' willingness to set aside the "ick factor" and collect samples, and the many clinicians and collaborators who (usually) diligently followed the shipping instructions to ensure safe delivery of packages of poop. Thank you also to the student workers for sparing me much of the "hands-on" time with those samples.

Finally, this work would not have been possible without my family and the unconditional love, patience, and support of my husband. I promise I will finally stop going to school.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professors Jan S. Suchodolski [advisor] and Jörg M. Steiner [co-advisor] of the Department of Small Animal Clinical Sciences, Robert C. Alaniz of the Department of Microbial Pathogenesis and Immunology, and Rosemary L. Walzem of the Department of Poultry Science and Graduate Faculty of Nutrition, Texas A&M University; and Craig G. Ruaux of the Department of Small Animal Medicine, Institute of Veterinary and Biomedical Sciences, Massey University.

The following researchers and clinicians provided fecal samples and clinical history for patients used in various sections reported in this dissertation: Karin Allenspach (Iowa State University), Amanda Blake (Texas A&M University), Francesca Bresciani (University of Bologna), Blake Guard (Texas A&M University), Al Jergens (Iowa State University), Olesia Kennedy (EPI4Dogs), Yuri Lawrence (Texas A&M University), Cyrus Parambeth (Texas A&M University), Linda Toresson (University of Helsinki and Evidensia Specialist Animal Hospital), Stefan Unterer (Ludwig Maximilian University), and Sara Wennogle (Colorado State University).

A tremendous amount of data from a canine gene expression study was made available to me, and has provided an excellent reference and context for interpretation of results. The gene expression study was done by Wilke et al. (2012).

All other work for the dissertation was completed independently by the student.

Funding Sources

The student's graduate studies were supported by a Merit Scholars Fellowship from the College of Veterinary Medicine, Texas A&M University, and a Lechner Scholarship from the College of Veterinary Medicine, Texas A&M University.

This work was also made possible in part by funding through the Texas A&M Genomic Seed Grant. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Texas A&M University AgriLife Genomics and Bioinformatics Service.

NOMENCLATURE

ABC	ATP-binding cassette
AHDS	acute hemorrhagic diarrhea syndrome
AIEC	adherent-invasive *Escherichia coli*
ARE	antibiotic-responsive enteropathy
CCECAI	canine CE clinical activity index
CD	Crohn's disease
CE	chronic enteropathy
CIBDAI	canine IBD activity index
CoA	coenzyme A
DI	dysbiosis index
EPI	exocrine pancreatic insufficiency
FDR	false discovery rate
FRE	food-responsive enteropathy
FXR	farnesoid X receptor
GC	gas chromatography
GIT	gastrointestinal tract
HGE	hemorrhagic gastroenteritis
HUC	histiocytic ulcerative colitis
IBD	inflammatory bowel disease
KEGG	Kyoto encyclopedia of genes and genomes
KO	KEGG ortholog
LC	liquid chromatography

LCFA	long chain fatty acid (approx. 14-18 carbons)
LDA	linear discriminant analysis
LEfSe	LDA effect size
LXR	liver X receptor
MCFA	medium chain fatty acid (approx. 8-14 carbons)
MS	mass spectrometry
m/z	mass-to-charge ratio
NPC1L1	Niemann-Pick C1-like 1 protein
OTU	operational taxonomic unit
PCA	principal component analysis
PCoA	principal coordinates analysis
PICRUSt	phylogenetic investigation of communities by reconstruction of unobserved states
PLE	protein-losing enteropathy
PLS-DA	partial least squares discriminant analysis
PPAR	peroxisome proliferator-activated receptor
PUFA	poly-unsaturated fatty acid
QIIME	quantitative insights into microbial ecology
RFA	random forest analysis
RT	retention time
RXR	retinoid X receptor
SCFA	short chain fatty acid (approx. <8 carbons)
SIM	selected ion monitoring
TAG	triacylglycerol

TCA	tricarboxylic acid cycle
TIC	total ion current
TLI	trypsin-like immunoreactivity
TOF	time-of-flight
UC	ulcerative colitis
VIP	variable importance in projection
VLCFA	very long chain fatty acid (approx. >18 or >20 carbons)

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1. INTRODUCTION

1.1 BRIEF OVERVIEW OF CANINE GASTROINTESTINAL PHYSIOLOGY

Gastrointestinal physiology varies significantly among mammals. Ruminants, for example, efficiently harvest nutrition by cultivating an ecosystem in their forestomach. The microbiota breaks down nondigestible plant fiber (e.g., cellulose), releasing absorbable nutrients and biosynthesizing digestible protein as part of their own survival, which can subsequently be absorbed by the host organism downstream in the GI tract. In contract to ruminants, other herbivores, such as the mouse, rabbit, and horse, are hind-gut fermenters. While they have a simple stomach, their cecum performs somewhat analogous functions as the rumen in the ruminant: the cecum is home to a diverse population of microbiota and nutrients are released from plant fiber that otherwise would not be accessible to the host. Rabbits in particular are known to practice coprophagia to absorb certain essential vitamins and nutrients that are generated by the microbiota in the hind gut, but require a second pass for more efficient extraction. The world of veterinary medicine exposes researchers to many blueprints for life, but dogs bear particular similarity to humans. Both species are omnivores, and although both lack the “fermentation vat” of ruminants, the presence of microbiota in the colon permits digestion of cellulose and concomitant release of nutrients.

Due to the general similarities between humans and dogs, in many cases what is known about human physiology is extrapolated to canine physiology. However, veterinary researchers must also be open to differences existing even between these seemingly similar biological systems. Publicly-available databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000) are utilized for interpretation of results, providing a contextual

framework of the interrelationships of metabolites and enzymes for example. While the interweaving networks of biochemical pathways represented by human metabolism are generally similar to other mammals, including the dog, it is worth noting that some biochemical relationships between compounds are governed by species-specific metabolic processes. For example, xylitol, a sugar substitute, is considered safe for people, but may cause a fatal release of insulin in dogs and subsequent hypoglycemia and/or liver failure. Where possible, information known to be applicable to the dog is presented, but the limited availability of canine-specific information must be kept in mind.

Our understanding of the function of the GI tract is continuously evolving. Most obviously the GIT serves as the site of nutrient assimilation necessary for life. The process begins with oral intake of food, which is mechanically broken down through mastication and in some species, mixing with salivary amylase (not present in the dog). In the stomach, chewed food is mixed with gastric juice, rich in hydrochloric acid, and is then called chyme. The acidity of chyme is neutralized upon entry into the duodenum, where it mixes with pancreatic juice, rich in bicarbonate and digestive enzymes, as well as bile from the gall bladder. Importantly, bile is composed primarily of bile salts, fatty acids, cholesterol, and phospholipids (Kristiansen et al. 2007). The orchestration of lipase secretion from the pancreas and bile from the gall bladder is critical for the emulsification of dietary lipids and lipid-soluble compounds (e.g., fat-soluble vitamins) for absorption in the intestinal tract. The amphipathic bile salts form mixed micelles with cholesterol, triacylglycerides (TAGs, also known as triglycerides), and other lipids, which overcomes the hydrophobicity of the lipids and permits pancreatic lipase to hydrolyze TAGs to monoacylglycerides and free fatty acids, which are amphipathic and thus can be absorbed by the epithelial cells of the intestine. Once inside the enterocyte, free fatty acids and monoacylglycerides re-esterify to form TAGs. In addition to lipase, pancreatic enzymes also include proteases (e.g.,

trypsin and chymotrypsin) and amylase to break down proteins and carbohydrates. Another important enzyme, enteropeptidase (also known as enterokinase) is produced by the enterochromaffin cells of the duodenum rather than the pancreas and serves to activate pancreatic trypsinogen to trypsin (Light and Janska 1989). Although enteropeptidase is a brush-border enzyme, the action of bile salts facilitates its dispersal into the intestinal lumen (Lentze et al. 1982).

Throughout the small intestine, the mucosal surface has microscopic finger-like projections known as villi. The villi greatly increase the surface area, increasing opportunity for absorption of nutrients. Each villus also contains a lacteal, or lymphatic capillary. The TAGs formed by re-esterification inside enterocytes are combined with phospholipids, esterified cholesterol, and apolipoproteins to form chylomicrons (Kindel et al. 2010). They may also contain fat-soluble vitamins A, D, E, and K. The chylomicrons are secreted through the basolateral membrane into the lacteal, where they flow as a component of lymph to the thoracic duct and enter the bloodstream at the level of the subclavian artery. Since lipids are inherently hydrophobic, this is a critical mechanism for transporting this important group of compounds through the aqueous bloodstream. In contrast to proteins and complex carbohydrates, lipids can enter the bloodstream via lacteals without first going through the portal vein and the liver.

The small intestine is also the site of absorption of amino acids, carbohydrates, and some vitamins. The last segment of the small intestine, the ileum, has some unique absorptive functions, particularly with regard to bile acids. After serving their function in forming micelles with dietary lipids within the intestinal lumen, bile acids are liberated when those lipids are absorbed by the enterocytes. The synthesis of bile acids *de novo* from cholesterol in the liver is metabolically expensive, so the ileum contains active transporters for recovery of bile acids from the lumen and only a small proportion of the bile acid pool fails to be recovered (Hofmann 1999). Resorbed bile acids are transported to the liver via portal circulation, eventually returning to the gall bladder for

re-secretion with bile. This process is known as enterohepatic circulation. Importantly, the bile acids that are not recovered are a means for elimination of cholesterol, since cholesterol is consumed in the synthesis of bile acids. Due to this relationship between bile acids and cholesterol, it is not surprising that bile acids play a role in regulation of cholesterol biosynthesis and overall whole-body cholesterol homeostasis (Vlahcevic et al. 1991). However, bile acid metabolism is a very active and complex field of study in its own right, and much is outside the scope of this current work.

The metabolic function of the large intestine was somewhat underappreciated until fairly recently, when the microbial inhabitants were found to be associated with health and disease. The importance of the GI microbiota and the metabolites they produce is discussed at greater length in the next section, but it is worth mentioning that some consider the microbiome to be an organ that possesses its own physiology and pathology (Baquero and Nombela 2012). As mentioned, in the dog, the colon is the primary site of microbial action within the GI tract. Thus, as part of digestion and nutrient assimilation, the colon is expected to be the site of absorption of metabolites and nutrients that are released by microbial fermentation or other enzymatic action. The small proportion of bile acids that escape recovery in the terminal ileum are largely deconjugated and dehydroxylated by the microbiota, which enhances passive uptake by colonocytes. Fiber, or cellulose, is a dietary component that remains largely unchanged as it traverses the upper GIT, but is important for colonic health. Cellulose is the major component of plant cell walls and is made up of glucose monomers with 1,4-β-acetal linkages. An enzyme capable of breaking that chemical bond is generally unknown in the animal kingdom, so metabolic action by the microbiota is required to release nutrients from these molecules. Through microbial fermentation of carbohydrates (Topping and Clifton 2001) and amino acids to some extent (Smith and Macfarlane 1996), short chain fatty acids (SCFAs) are released, which are the main source of energy for

colonocytes. SCFAs were also recently shown to influence transport and metabolism of phenolics by Caco-2 cells (Van Rymenant et al. 2017), suggesting that production of one group of metabolites may influence the host's ability to utilize other metabolites. Beyond our incomplete understanding of the effects of the colonic microbiota, it is known that the colon also provides the function of water and electrolyte extraction from the digesta, with the remaining material passing into the rectum for storage until defecation.

Another perhaps less obvious function of the GIT is as the interface between the external environment and the immune system. While the importance of the gastrointestinal tract as an immune organ cannot be overstated, and it is already established that some metabolites in the GIT have anti-inflammatory activity (Aldini et al. 2014; Miquel et al. 2015), the vast majority of immunologic mechanisms pertaining to the GI tract will not be discussed in detail herein.

While gastrointestinal physiology overall is far more complex than can be fully described here, and research to enhance our understanding is ongoing, this overview aims to provide a basic framework for interpretation of the research presented within this dissertation.

1.2 THE CANINE GASTROINTESTINAL MICROBIOTA

The intestinal microbiota is comprised of bacteria, fungi, protozoa, and viruses living within the gastrointestinal tract. The coevolution of the gut microbiota with mammalian host species has resulted in an intimate commensal relationship and codependence to maintain homeostasis. The limits of influence that the microbiota exerts on the host have not yet been fully determined. Prior to the development of DNA sequencing methods and bioinformatics, exploration of the gastrointestinal microbiota was limited by cultivation techniques, which neglected many of the inhabitants and likely failed to accurately mirror metabolic behavior *in vivo*. DNA-based techniques do not require the ability to sustain the life of a given species in order to detect it, so

characterization of the composition of the intestinal microbiota has improved with the emergence of high throughput sequencing platforms. Unfortunately, these techniques applied to the bacterial 16S rRNA gene continue to neglect non-bacterial inhabitants and like any method are not without inherent bias, but 16S rRNA gene sequencing seems to be the most popular method for characterizing the microbiota, even if it is not comprehensive. Fungal inhabitants have been characterized in the dog using fecal samples and 18S rRNA sequencing (Foster et al. 2013). Suchodolski et al. (2008) used clonal libraries to show that the composition of the microbiota varies at different sites within the canine GIT, yet the vast majority of studies available have used fecal samples as a representative surrogate for the entire GIT.

At the taxonomic level of phylum, the composition of the fecal microbiota of humans and dogs exhibit similarities, with the predominant phyla being Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria (Honneffer et al. 2014; Hooda et al. 2012; Jandhyala et al. 2015; Swanson et al. 2011; Handl et al. 2011). While the physiological function of the microbiota was touched upon in Section 1.1, the importance of the microbiota in maintaining health is most profoundly demonstrated by the plethora of literature comparing the microbiota of diseased patients to that of healthy controls. In dogs, several studies have shown significant changes associated with both acute and chronic gastrointestinal disease (Suchodolski et al. 2012a; Vazquez-Baeza et al. 2016; Wilke et al. 2012; Suchodolski et al. 2010; Suchodolski et al. 2012b; Honneffer et al. 2015). In humans, changes in the microbiota have also been associated with a wide range of extra-GI diseases: arthritis (Costello et al. 2015), autism (Rosenfeld 2015), cardiovascular disease (Z. Wang et al. 2011), and renal disease (Ramezani and Raj 2014) as well as potentially demonstrating behavioral influence in experimental animal models (Collins et al. 2013; Cryan and Dinan 2012). In the vein of the GIT as an immune organ, the role of the gut microbiota in immune-mediated inflammatory diseases was also recently reviewed (Forbes et al.

2016). In spite of these associations, in many cases, a direct causal relationship has not been established and work is ongoing to understand the relationship between disease and changes in the microbiota, or dysbiosis. There are some situations where the evidence is strong that the microbiota plays a role in the pathogenesis of some diseases. For example, adherent-invasive *Escherichia coli* (AIEC) play a major role in the pathology of granulomatous colitis (also called histiocytic ulcerative colitis, HUC) of Boxer dogs, where elimination of AIEC correlated strongly with clinical remission of disease (Mansfield et al. 2009). Other well-known enteropathogens include *Campylobacter jejuni* and *Salmonella* (S. L. Marks et al. 2011). Furthermore, while *Clostridium perfringens* has been shown to inhabit the GIT of healthy dogs (Minamoto et al. 2014), recent work has shown a strong association between the presence of a specific genotype of *C. perfringens* (containing a pore forming cytotoxin gene called *netF*) and cases of necrotizing enterocolitis of foals as well as acute hemorrhagic diarrhea syndrome (AHDS, formerly hemorrhagic gastroenteritis, HGE) in dogs (Gohari et al. 2015; Unterer et al. 2014). Specific canine gastrointestinal diseases, including AHDS, will be discussed further in Section 1.3.

Whether or not a causal relationship is established between the microbiota and pathogenesis of disease, there would be much to understand about where, why, and how the presence (or absence) of microbial species causes disease, and how we might eventually leverage our understanding in order to improve diagnostics and therapeutics for our patients. This requires moving beyond identification of which microbial species are present, instead considering what metabolic consequences they have. One of the key reasons for this is the functional redundancy of the microbiota. For example, the ability to ferment carbohydrates or amino acids and release SCFAs as described above may not be ubiquitous among GI bacteria, but several genera within Clostridia possess this ability in their functional repertoire (Macfarlane and Macfarlane 1997), allowing a change in composition without necessarily concomitant change in function. The

converse is also true: many bacteria can change their substrate utilization depending on what is available, resulting in a change in function without any change in microbial composition. A model has been developed for the mechanism by which *E. coli* can accomplish this regulation of metabolism based on metabolic fluxes (Kotte et al. 2010). However, if the biochemical milieu of a microbial ecosystem were analyzed for all of the metabolites present, perhaps a snapshot of the net effect of the community's functional capacity could be determined. In Section 1.4, the field of metabolomics is described, which aims to capture the biochemical composition of a sample.

1.3 GASTROINTESTINAL DISEASES IN DOGS

Clinical signs commonly associated with gastrointestinal disease, such as vomiting, diarrhea, or weight loss, are nonspecific due to the wide variety of conditions that can cause these complaints. Gastrointestinal diseases are often further characterized by considering the time course of illness (acute or chronic), success of response to various treatments (i.e., antibiotic responsiveness, food responsiveness), as well as by quantification of biomarkers or other diagnostics that may or may not be pathognomonic for a disease.

Chronic enteropathy (CE), acute hemorrhagic diarrhea syndrome (AHDS), and exocrine pancreatic insufficiency (EPI) are three very different diseases that affect the GIT and typically present with clinical signs of gastrointestinal disease. Although CE and EPI may occur concurrently, EPI can be excluded by a serum trypsin-like immunoreactivity (TLI) concentration within the reference interval. A serum cTLI concentration < 2.5 μg/L is highly suggestive of EPI, but concurrent CE is far more difficult to rule out. The clinical presentation of AHDS is very distinct due to the peracute time course and accompanying clinical signs.

1.3.1 Chronic enteropathy

A discussion of chronic enteropathy cannot occur without a clarification of terminology in order to prevent misunderstandings due to inconsistencies in disease definitions. For dogs with chronic (duration > 3 weeks) gastrointestinal signs, it is largely agreed that some combination of genetic predisposition, environmental factors (potentially including diet), and the GI microbiota lead to aberrant immune function, but a precise terminology for this disease has not been established. Dandrieux (2016) summarized that using the term "inflammatory bowel disease", which is already used in human medicine to encompass Crohn's disease (CD) and ulcerative colitis (UC), may be disadvantageous because of important differences between the management of the disease in humans and dogs. Examples of these profound differences between dogs and humans include the routine use of biologics (e.g., humanized antibodies directed against tumor necrosis factor) and the frequent necessity of surgical therapy in humans with IBD, compared to a fairly high rate of success with dietary management alone in dogs. In fact, the term IBD at face value suggests a prominent inflammatory component, and in many cases, dogs with chronic GI signs do not undergo biopsy to confirm an inflammatory infiltration and rule out other potential non-inflammatory causes. Furthermore, the clinical signs associated with disease are indicative of aberrant function, but the presence of inflammation is not necessarily a prerequisite to the pathophysiology.

To address cases where chronicity of GI disease has been established, but inflammation can only be presumed due to a lack of histopathological assessment, the more general term "chronic enteropathy" is favored. Literally implying a gastrointestinal problem that has persisted beyond an acute issue, this term could be criticized as extremely broad. Fortunately, there seems to be agreement that a diagnosis of "chronic enteropathy" implies exclusion of other diseases that have more specific criteria, and nuances of the clinical signs as well as available diagnostic tests

may easily exclude other causes of vomiting and diarrhea. Dandrieux further describes the retrospective assignment of dogs with CE to different subgroups based on their response to treatment. Dogs with food responsive enteropathy (FRE) are recognized following improvement of clinical signs during a dietary intervention trial, often an exclusion diet, a hydrolyzed protein diet, or a novel protein diet. Unfortunately, scientific evidence is inconclusive regarding which diet would be most beneficial for a given patient, and failure to improve on one diet may not definitively rule out the possibility that a different diet would have been associated with a different outcome. Nonetheless, it is presumed that either approach (i.e., use of a hydrolyzed or novel protein) will decrease or eliminate the antigenic effect of the diet, thus assuaging the assumed pro-inflammatory feedback loop that perpetuates CE, and improvement is often seen over a matter of days. Of the dogs with CE that do not improve with a faithfully executed dietary trial, endoscopy is generally recommended to confirm the presence of and characterize the inflammation and rule out other causes of chronic GI signs, such as GI lymphoma.

Medical management of CE in dogs that do not respond adequately to diet alone often proceeds with an antibiotic trial, using metronidazole, or more frequently, tylosin. Recent studies have demonstrated a profound effect of antibiotic therapy on the composition of the microbiota as well as the biochemical environment (metabolome) within the GIT when metronidazole was administered to healthy dogs for two weeks (Suchodolski et al. 2016). It is unclear exactly how this or other antibiotics are improving the clinical signs of some dogs with CE, but dogs that have an apparent dependence on antibiotics to control clinical signs are classified as having antibiotic-responsive enteropathy (ARE). Importantly, these dogs typically relapse when the antibiotic is discontinued. This is distinct from infectious causes of chronic diarrhea, including granulomatous colitis of Boxers mentioned in Section 1.2, wherein clinical improvement correlated with clearance of an enteropathogen, such as AIEC.

Finally, dogs with CE that have failed to respond to diet and antibiotic treatment, or relapse in spite of ongoing treatment, are generally treated with immunomodulatory drugs. These dogs arguably are most similar to the human form of IBD, and in fact some clinicians reserve the term "IBD" for these dogs that have failed dietary and antibiotic trials. Some of these dogs are non-responders and continue to do poorly in spite of increasing doses of immunosuppressants, but the review by Dandrieux (2016) cites success rates ranging from 25-100% with variable treatments and duration of follow-up.

Another important subgroup of CE is protein-losing enteropathy (PLE). This subset of patients is defined by the presence of hypoalbuminemia in addition to CE, without evidence of non-GI causes of protein loss. Because of the severity and rapid progression of this disease in many patients, these dogs are often treated aggressively with a multi-modal approach, so if they improve, categorizing them as FRE, ARE, or IRE is often impossible.

Regardless of the subcategorization of CE, there are two published scoring systems in use for grading severity of disease, the canine IBD activity index (CIBDAI) (Jergens et al. 2003), and the canine chronic enteropathy clinical activity index (CCECAI) (Allenspach et al. 2007). Jergens et al. (2003) found a strong correlation between improved histology, CIBDAI score improvement, and decreased serum measurements of C-reactive protein (CRP) after medical therapy. Allenspach et al. (2007) suggested that the clinical importance of the CCECAI is the use of hypocobalaminemia and hypoalbuminemia as negative prognostic indicators. While both scoring systems may have utility to the clinician making treatment decisions and assessing response to therapy, these parameters do little to inform the researcher about the pathogenesis of the disease, and new diagnostic panels and indices are needed to better elucidate the biological changes that occur during disease.

Among those biological changes thought to be relevant to disease progression, the term "dysbiosis" invariably arises in discussions of CE and chronic GI disease. Dysbiosis can be defined as an abnormal change in the composition of the microbiota. Because folate and cobalamin are absorbed by the proximal and distal small intestine, respectively, and bacteria in the gut may produce folate and consume cobalamin, serum measurements of these B vitamins are sometimes used to assess small intestinal health status. Decreased serum levels could be due to mucosal damage of the small intestine, hindering absorption. However, an abnormal microbial composition could also result in increased luminal production of folate and consumption of cobalamin. Although serum concentrations of folate and cobalamin are used to guide treatment to an extent, it appears likely that basing a diagnosis of dysbiosis solely on measurement of these two parameters in serum would involve unwarranted assumptions in many cases. Recently, a quantitative polymerase chain reaction (qPCR) panel has been developed to use a few key taxa of the fecal microbiota to establish a dysbiosis index (AlShawaqfeh et al. 2016). The dysbiosis index (DI) was trained using fecal DNA from 43 healthy dogs and 64 dogs with CE and validated with an additional 242 healthy dogs and 108 diseased dogs. Thus the DI may be particularly useful for assessing the shift in the microbiota that occurs most commonly with CE, but it may under- or over-estimate the degree of microbial dysbiosis in diseases with shifts in microbial populations other than those included in the panel. However, if the bacterial groups in the panel are core components of a healthy canine gut microbiota, the interconnectedness of the entire gut ecosystem would suggest that some shift in the DI should be detected. The clinical utility of the DI in guiding treatment decisions has not yet been determined. Unfortunately, the DI may also be heavily swayed by a recent history of antimicrobial use. While this gives credence to the idea that an upset microbial composition (known to occur with antibiotic usage) results in an increased DI, it also means that dogs with CE who have received antibiotics recently are not good candidates for a

meaningful assessment of dysbiosis. An additional condition known to increase DI is exocrine pancreatic insufficiency (EPI) (Isaiah et al. 2017). This disease is discussed in greater detail in Section 1.3.3.

1.3.2 Acute hemorrhagic diarrhea syndrome

Acute hemorrhagic diarrhea syndrome (AHDS) is a severe, sudden-onset syndrome affecting the canine gastrointestinal tract, and is characterized by vomiting and bloody diarrhea, but is also often associated with lethargy, anorexia, and abdominal pain. This syndrome was previously known as hemorrhagic gastroenteritis (HGE), but recent work has revealed that the stomach is not involved (Unterer et al. 2014), and the more appropriately descriptive AHDS terminology is now preferred. The etiology of AHDS is unknown, though there is a strong suspicion for a role of a strain of *Clostridium perfringens*, either as a causative agent or as a frequent opportunist secondary to the disease, since it was detected much more frequently in diseased dogs than control dogs (Suchodolski et al. 2012b). However, this is complicated by the prevalence of *C. perfringens* among healthy dogs (Minamoto et al. 2014) and the fact that samples are collected after the onset of diarrhea, so proof of induction of disease is difficult. Furthermore the enterotoxin *cpe*, proposed as the relevant agent for diarrhea in dogs, may also be present in healthy dogs (Silva and Lobato 2015; S. L. Marks et al. 2011), and the clinical course of infection may vary from self-limiting to fatal. In short, the presence or absence of enterotoxins does not appear to be a relevant factor in choosing therapy or determining prognosis of the disease (Busch et al. 2015).

The histopathological changes associated with AHDS suggest that the initial insult invokes a massive peracute increase in intestinal permeability with acute mucosal hemorrhagic necrosis, subsequent extravasation of fluid, plasma proteins, and red blood cells into the intestinal lumen. For an excellent article summarizing the clinical course of a large cohort (n=108) of dogs

with AHDS, the reader is referred to a recent paper by Mortier et al. (2015). In regards to the intestinal microbiome, the potential prevalence of bacteremia and a corresponding necessity for antibiotic therapy was recently studied (Unterer et al. 2015), and it was ultimately determined that bacteremia based on blood culture was equivocal between patients with AHDS and control dogs. While this does not mean that dogs with sepsis should not be treated with antibiotics, it does suggest that antibiotics are not an appropriate prophylactic treatment in dogs with AHDS. This is largely based on the fact that animals with positive blood cultures and those with negative blood cultures were equivocal in terms of outcome and clinical parameters, so bacteremia (in the absence of signs of sepsis) does not necessarily suggest any benefit of antibiotic therapy, and in fact, considering the previously described disturbance to the microbiome induced by antibiotic therapy, their injudicious use may be detrimental to the patients.

One additional aspect regarding AHDS is that patients typically recover very quickly, often only with supportive care. Although not yet confirmed, this may suggest that the physiological responses to such a severe mucosal insult in a normal dog may be intact, and perhaps this is distinct from a dog with chronic enteropathy, who may have an underlying genetic predisposition. On the other hand, the fact that a given animal develops AHDS may indicate some other predisposition or inciting factor that parallels, or mimics the process that is ongoing in chronic enteropathy. However, another possibility is that the comparison of histopathology is misleading, and the etiology of mucosal damage (and perhaps the sequelae in terms of the effect of the luminal contents) is completely dissimilar between AHDS and chronic enteropathies. Thus, comparison of the microbiome and metabolome may yield additional insight into these diseases.

1.3.3 Exocrine pancreatic insufficiency

In contrast to AHDS and CE, exocrine pancreatic insufficiency (EPI) is characterized by a functional defect rather than a clinical presentation or histopathological finding, and the

pathogenesis generally is better understood. The pancreas consists of both endocrine and exocrine functions. The endocrine pancreas is responsible for critical functions such as insulin production for regulation of body-wide glucose homeostasis. The exocrine pancreas is the primary source of digestive enzymes that are secreted into the GI tract for digestion of dietary components, as briefly described in Section 1.1. Exocrine pancreatic function is quantitatively assessed by measuring the serum concentration of trypsin-like immunoreactivity (TLI). During health, a small amount of trypsinogen and other enzymes and zymogens leak into the vascular space. The exocrine pancreas also synthesizes and secretes pancreatic lipase, an important enzyme for fat digestion and assimilation.

Causes of EPI include pancreatic fibrosis, atrophy (most likely due to immune-mediated destruction), or much less frequently, hypoplasia. Clinical signs usually do not ensue until more than 90% of exocrine pancreatic function has been lost. Regardless of the cause of the decreased exocrine pancreatic tissue, the result is impaired secretion of pancreatic enzymes into the lumen, and subsequent maldigestion of dietary lipids, carbohydrates, and proteins by virtue of the decreased lipases, amylase, and proteases entering the proximal duodenum. Without adequate lipase being present, small dietary lipids may still be solubilized by bile acids, but lipid assimilation is greatly impaired, also affecting assimilation of lipid-soluble vitamins and essential fatty acids. The composition of the luminal digesta then is greatly altered from normal, which provides an abnormal substrate for the GI microbiota, and subsequent dysbiosis develops, adding to the dysfunction.

Fortunately, although treatment is lifelong and the cost of exogenous enzymes may be a consideration for some owners, many dogs with EPI clinically respond well to enzyme replacement therapy. While all owners and clinicians would be happy to have resolution of clinical signs, it is not clear whether this corresponds to normalization of all metabolic disruptions caused

by the disease. Enzyme replacement therapy is expected to supply the enzymes to the lumen, but importantly, this will not change the serum TLI concentration. Thus, assessing efficacy of treatment is limited to resolution of clinical signs. It is possible that analysis of fecal metabolites may be helpful in assessing the extent of both dysbiosis and dysmetabolism that are part of the pathogenesis of EPI.

1.4 METABOLOMICS

Metabolites can loosely be defined as biochemicals that result from anabolic and catabolic processes within an organism. While this includes amino acids and even short peptides, it does not encompass large biomolecules such as proteins. A metabolomics approach, then, is an attempt to survey a sample broadly for the presence and abundance of metabolites. Urinalyses and serum chemistry panels could be considered targeted metabolomics panels – measuring the concentrations of compounds such as creatinine, glucose, cholesterol, and blood urea nitrogen. In the past two decades, a much broader approach has been utilized: untargeted metabolomics (Kell and Oliver 2016). In comparison to analysis for a specific set of metabolites, untargeted metabolomics aims to detect signals from compounds both known and unknown, and regardless of whether or not they are expected. This makes untargeted metabolomics an excellent approach for biomarker discovery, as it isn't limited by what is currently known about a given phenotype or how a phenotype is expected to affect a biological sample.

1.4.1 Untargeted metabolomics platforms

The breadth and quality of coverage of a given sample type by untargeted metabolomics may depend on the platform(s) used, and each platform has limitations in the types and/or sizes of molecules it can detect. The two most common approaches for the characterization of the metabolome are nuclear magnetic resonance (NMR) analysis and mass spectrometry (MS). NMR

has the advantage of being non-destructive, so the sample can be recovered after analysis and used for additional testing. NMR also has the advantage of being quantitative; for example, every proton generates the same integrated peak area regardless of the molecule it is part of. MS, on the other hand, consumes the sample being analyzed, and the magnitude of signal does not correlate to the quantity of different compounds (but importantly does allow comparison of signal for the same compound across different samples). However, the sensitivity of MS greatly surpasses that of NMR, making it by far the more popular choice for metabolomics experiments and the only method that will be discussed here further.

Mass spectrometry is a method of detection, and there are many different types of MS that can be used, as well as different front-end components. Quantifying any target out of a complex matrix requires both a way to separate the target from matrix and other interfering components, and a method to detect and quantify a signal specifically from the target. Chromatography is a term generally used to describe a process of separating a mixture into distinct components, usually by using a variety of chemical properties of the components to spend variable time periods in a stationary or mobile phase. For metabolomics, the two most common types of chromatography are liquid chromatography (LC) or gas chromatography (GC). Components that spend more time in the movement phase will move over a distance more quickly, while components that spend more time in the stationary phase will take longer to pass the same distance. The stationary phase in most types of chromatography is called a column, and the movement phase is a solvent (for liquid chromatography) or carrier gas (for gas chromatography). GC-MS-SIM is the abbreviation for gas chromatography-mass spectrometry-selected ion monitoring, and this technique will be described in greater detail to demonstrate the concepts of chromatography and detection that are critical to metabolomics. GC is a method to physically stratify the mixture, while MS serves as the

detection method. The sensitivity of detection is enhanced if the instrument is set only to quantitate ions relevant to the target, so SIM is an approach to limit the ions that are being monitored.

Gas chromatography stratifies components of a mixture on the basis of volatility and interaction with the stationary phase of the capillary column versus the gas phase (typically helium flowing at a rate that allows interaction of the compounds with both the column and the gas). As a generalization, compounds that are smaller or more volatile spend more time in the gas phase of the column and thus are carried through more quickly, while larger, less volatile compounds spend more time interacting with the solid phase and move through more slowly, though the specific properties of the column used can affect the affinity of different compounds to the stationary phase. The entire column is housed within an oven, so after the sample is injected, the oven can be programmed to run a gradient. This can help increase separation of different compounds that come off early and speed the process of eluting slower compounds. With various parameters on the GC, the analyst can fairly well devise a protocol to separate different compounds from each other, with the caveat that certain differences (e.g., conformation differences of chemical isomers) are much more difficult to separate with simple gas chromatography. Also, large molecules may not become volatile enough to move appreciably in the gas phase, so larger/heavier compounds do not reach the detector.

Liquid chromatography operates on the same principles as GC, but with an important difference that the solvent controlling the movement phase can be altered over time. This provides an additional dimension for separation of compounds since the relative affinity of the compounds to the column at the beginning of the run (e.g., when 100% solvent A is passing through) can be changed (e.g., by switching to 70% solvent A and 30% solvent B). The properties of the chromatography column can also be changed, yielding additional options such as reverse phase (RPLC) and hydrophilic interaction (HILIC). There are other advantages associated with LC such

as not requiring volatility as is required with GC, which often obviates the need to derivatize compounds prior to analysis. This process in the context of GC is described later.

As the compounds separate from each other and reach the end of the column (theoretically in a series rather than mixed together), they flow into the mass spectrometer. For GC, perpendicular to the flow of the sample, there is an electron beam generated from a filament and these electrons impact the molecules, causing the vast majority to ionize and fragment. For LC, the desolvation of compounds as they enter the detector results in ionization, and a portion of the compound enters the detector without fragmentation. Knowing the molecular ion peak, or [M+], is one of the most useful pieces of information for identifying an unknown compound. With either GC or LC, some of the molecules will fragment. The fragmentation is not random; rather the nature of the chemical bonds within the molecule dictates which are most likely to break, and potentially subsequent rearrangement within the residual fragments. A single molecule may break in any number of ways, but across a population of molecules of the same compound, a characteristic pattern of fragments will be associated with a given compound. This fragmentation pattern is called the mass spectrum. These fragments, each of which has a certain mass-to-charge ratio (m/z), are referred to as daughter ions, then flow into the quadrupole, which is a series of magnets that together can filter certain m/z fragments. In scan mode, the quadrupole scans over the entire mass range (typically 50-600 m/z) very rapidly. Although no m/z fragments are specifically excluded, at any instant only one m/z is allowed through. As the fragments pass the filter they reach the detector, which utilizes an electron multiplier to generate a signal proportionate to the number of fragments hitting the detector. Coordination of the quadrupole with the detector allows rough quantification of every m/z in the scan range. The signal over time will trace a total ion current chromatogram (TIC), which represents the sum of all fragments that have reached the detector. The peaks on the chromatogram correspond to compounds moving through, and the time it takes

to elute after injection is called the retention time (RT). If two compounds still co-elute, the mass spectrum at their RT will reflect the superimposition of the characteristic fragmentation patterns of both compounds.

Because biological matrices can be complex, and several distinct compounds could have the same nominal mass and similar mass spectra, more advanced MS instruments use time-of-flight (TOF) to gain much higher mass accuracy for the molecular ions and the fragments. It is expected that relatively few chemical formulas are consistent with a specific, extremely accurate mass determination, and by predicting the fragmentation structure of those compounds, a "best-fit" decision can be made to assign the unknown to a specific metabolite. Another advanced approach, though utilized when targeting certain compounds, harnesses repeated MS filtering steps. These instruments, sometimes referred to as "triple quad" for having three quadrupoles rather than a single quadrupole, or "tandem MS" for the sequential MS steps, can be operated in different modes. As an example, they use the first quadrupole to fragment compounds, a second quadrupole to select only certain fragments emitting from the first quadrupole and further fragment them, and a third quadrupole to detect the subsequent fragments. For two co-eluting compounds, even if their initial fragmentation pattern is similar, it is likely that secondary fragmentation of one of the primary fragments can be used to distinguish the two compounds. An additional instrument type has combined the triple quad with TOF and is referred to as a Q-TOF. These instruments are capable of the highly sensitive tandem MS quantitation, but also access high mass accuracy analyses. Importantly, even with high mass accuracy or tandem MS methods, there is always a possibility of incorrect assignment of metabolite identity. Thus once untargeted metabolomics has potentially revealed some metabolites of interest, it is critical to validate the findings with authentic standards (if available). Finally, if it is desired to use the metabolite as a biomarker, a targeted,

quantitative method may be required so a reference interval can be established and individual samples can be compared to this reference interval.

1.4.2 Targeted metabolomics and quantitation

For untargeted metabolomics, the most ideal analysis may involve multiple platforms. Analyzing the same sample by LC-MS and GC-MS may enhance both the mass range that is accessible as well as the diversity of compounds that can be accurately detected. However, once there is a specific subset of molecules that are of interest, the focus becomes accurate and sensitive quantification of those specific compounds. Analysis for a specific subset of molecules is a type of targeted metabolomics. Since the targets are known, their fragmentation spectra can be characterized and the relationship between signal and quantity can be calibrated if a pure standard can be obtained. Additional techniques can then be employed to enhance detection of desired compounds.

In highly complex matrices, complete chromatographic resolution of all compounds may be difficult to obtain. Once the fragmentation pattern of an isolated compound of interest is known, certain fragments can be chosen that are unique to the target compound and generate a strong signal. With access to a triple quad or Q-TOF instrument, the secondary fragmentation pattern associated with a primary fragment can also be characterized. However, subsequent descriptions will assume use of a single quadrupole instrument. The quadrupole is used to filter out ions except for those associated with the target compounds, or “selected ion monitoring” (SIM). Instead of scanning repeatedly over the entire possible mass range, the quadrupole only cycles through all the ions of interest. To further enhance sensitivity, the set of ions being monitored can be changed at different times after injection of the sample. These periods are referred to as “SIM windows” and as long as the target compounds elute during their designated SIM window, the relevant ions will be detected. Maintaining the SIM windows relative to elution time after cutting the column

requires “retention time locking” (RTLock). This involves a one-time calibration of the relationship between carrier gas pressure and retention time. From that equation, when the column length is changed, the pressure can be adjusted to maintain consistent RTs.

When using SIM, selection of ions is extremely critical. For each compound of interest, a target ion must be selected. This is the ion that will be used to quantify the compound relative to a calibration curve. Additional ion(s) are selected as qualifying ions. Since the relative quantity of fragments in the mass spectrum from a compound are characteristic for that compound, the ratio of different fragments can be used to confirm the identity of the compound, particularly in conjunction with the characteristic retention time. Failure to match the expected ratio may indicate another compound co-eluting in the sample and quantitation may be incorrect. Proper mass assignment is also critical: while publications typically report the nominal mass of a fragment (e.g., a CH_3 group has m/z = 15), the actual mass of the fragment is slightly greater. Of note is that this is distinct from the concept of “molar mass,” which takes into consideration the isotopic composition of a very large number of molecules. Interestingly, this is a manifestation of Einstein’s famous equation $E=mc^2$ – meaning that the energy of these fragments results in a mass greater than simply the sum of protons and neutrons. As an example, typical fragments for sterols may be nominally 219 or 334, but the quadrupole selects for 219.3 and 334.4 (±0.2). An example of a TIC from a GC-MS-SIM analysis on a fecal sample is shown in Figure 1.

A calibration curve and internal standard (ISTD) are used to transform the signal generated by a specific target ion into a concentration. The target ion for the ISTD and the target ion for an analyte of interest have a fundamental relationship in terms of relative response of the detector. Once the calibration is established to define that relationship in terms of a concentration, it is quite stable over time so recalibration only needs to be done infrequently (as needed, though the calibration for every compound should be verified with every run).

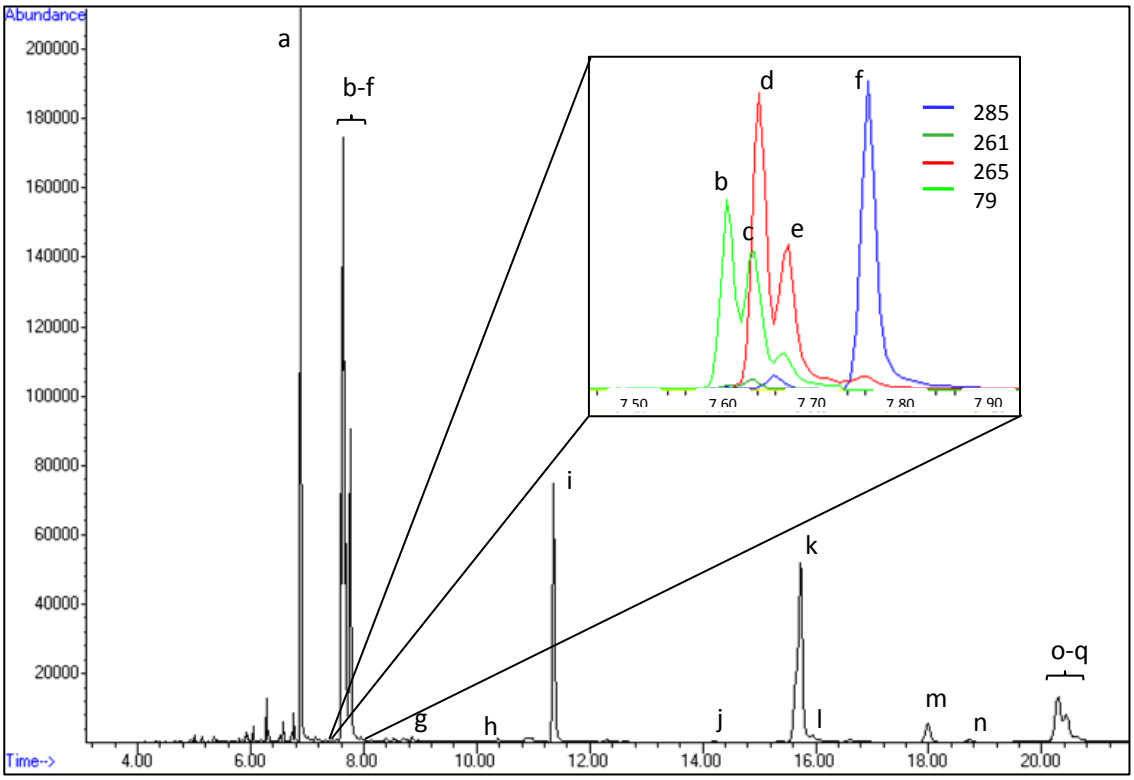

Figure 1. Total ion current (TIC) chromatogram for a typical canine fecal sample. Inset shows individual ion currents for a subset of fragments associated with fatty acids. Compounds identified by letters (retention time in minutes) are **a**: palmitic acid (6.9); **b**: linoleic acid (7.6); **c**: α-linolenic acid (7.6); **d**: oleic acid (7.7); **e**: *cis*-vaccenic acid (7.7); **f**: stearic acid (7.8); **g**: gondoic acid (8.7); **h**: erucic acid (10.2); **i**: d4-cholestane (11.4); **j**: coprostanol (14.2); **k**: cholesterol (15.7); **l**: cholestanol (15.8); **m**: campesterol (18.0); **n**: stigmasterol (18.8); **o**: fucosterol (20.3); **p**: β-sitosterol (20.3); **q**: sitostanol (20.7). Obtained under parameters described in Section 6.

1.4.3 Metabolomics and feces

Fecal matter represents the net result of diet, host GI function, and the metabolic effects of the microbiota. Given the relative ease and non-invasiveness of collection, using feces as a diagnostic sample holds great potential, while also presenting some significant challenges. First, it is critical to note that the composition of biochemicals within the gastrointestinal tract is the result of the collective metabolic activity of the microbiota as well as the host, and may be significantly shaped by dietary intake. For example, when comparing the fecal metabolite profile of dogs with chronic enteropathy to that of healthy control dogs as described in Section 5, a metabolite identified as famotidine was detected in a subset of diseased dogs. Frequently prescribed to dogs with gastrointestinal disease, this finding is not surprising, but it demonstrates that oral intake of metabolites (or their precursors) does in fact have an effect on the fecal metabolomic profile. This must always be considered a confounding variable when diet is not standardized, which is nearly always the case for clinical research where dietary choice is largely driven by patient and owner factors. To further complicate this facet of research, information pertaining to diet is often scarce in clinical work. Within an owner questionnaire, a query of the dietary history for their pet may result in answers such as “dry” or “dog food”. Although honest, it is unlikely that this level of detail will allow researchers to distinguish the effect of corn- versus soy-containing diets, nor have the data to consider whether concentrations of some metabolites are largely driven by the ingredients of the diet. This may become especially relevant with regard to questions being raised in the research community about the efficacy of novel-protein versus hydrolyzed protein diets.As a further demonstration of how the metabolomic composition of feces can be affected, measurements on material taken from the human colon has shown a relationship between the presence of starch and the bacterial propensity to ferment amino acids (Smith and Macfarlane 1996). Bacteria require a nitrogen source, and mucus, dietary protein, and enterocyte

turnover are all nitrogen sources for the gut microbiota. Using culture techniques where carbohydrate sources were excluded, Smith and Macfarlane (1996) showed a preference on the part of the fecal microbiota for peptides over free amino acids, with SCFAs and branched chain fatty acids (BCFAs) as the products. While SCFAs are usually considered to be the product of carbohydrate fermentation, it demonstrates the resourcefulness of the microbiota that SCFAs can also be produced from peptides and amino acids. In addition, these data demonstrated that adding fermentable carbohydrate altered the profile of SCFAs produced, and decreased net ammonia production (which suggests decreased bacterial amino acid degradation). This highlights the potential utility of using feces as a surrogate sample for the entire GI tract. Since the metabolomic composition of feces involves tremendously complex biochemical interrelationships, and the different locations within the GI tract carry out different metabolic reactions in physiologically normal organisms, it is very likely that an abnormal process at a proximal point of the GI tract will result in material of abnormal composition arriving at the next distal point, such that the net metabolic result in the feces very well may be an altered concentration of a given metabolite compared to that of healthy feces. Furthermore, comparing concentrations of multiple compounds simultaneously may better reveal the changes to the metabolic processes occurring, as the deviation from normal may be the combination of many small changes.

1.4.4 Metabolomics and data analysis

Untargeted metabolomics invariably produces large data sets, with many features (metabolites) detected in each biological sample. The number of features in a targeted metabolomics assay may vary greatly, but by definition is more than one feature. With multiple features to explore, multivariate analyses become a useful tool for data exploration. In subsequent sections, some of these methods are used, especially to help visualize complex data sets. One example is principal components analysis (PCA). A PCA plot is a way to easily see the spread of

data and check for outliers in the global profile. The composition of a sample in a data set is summarized in relation to all other samples – creating a spatial arrangement such that samples most similar to each other are in close proximity, while samples that are very dissimilar are far from each other. PCA is unsupervised – which means it does not take into consideration what group the sample belongs to (e.g., healthy versus diseased), rather the pattern of separation must be found purely by the data themselves. This also requires that the within-group variation is sufficiently less than between-group variation (Worley and Powers 2013). Therefore, if one plots a two-group data set with PCA and the two groups are completely distinct, the combined features of the data set successfully distinguish the two groups. On the other hand, where there is overlap between the two groups, it would suggest that there is still enough variability that the features in the data set are not adequate to completely distinguish sample groups.

In comparison, partial least squares projections to latent structures (PLS-DA) produces similar-looking plots, but the algorithm begins with the classification into groups as assigned by the data set, which makes this a supervised method. The algorithm can further be queried to determine which of the features in the data set are most important to establishing the separation, or the variable importance in projection (VIP) score.

Random forest analysis (RFA) is another form of multivariate analysis. This method uses the different features in the data set in a random way to classify and determines the error in classification. Repeating this over many permutations (bootstrapping), certain features will eventually be observed as having a greater impact on the accuracy of classification. Therefore, this method also produces a ranking of the importance of the features, defined by the mean decrease in accuracy. Since PLS-DA and RFA both aim to rank the importance of features in the data set, but have very distinct algorithms to achieve this, comparing the results of the two methods can

provide greater support for features that are ranked as highly important using both methods rather than only one of the two methods.

An interesting feature of multivariate analyses is that different data sets associated with the same samples can be combined. This method is utilized in Section 2, where the 16S rRNA sequencing data were combined with the metabolomics data from samples along the canine GIT to search for correlations between microbial composition and metabolites. However, multivariate analyses are particularly sensitive to the scale of data. In other words, the most abundant (highest concentration) compounds or sequences will inherently carry more weight than low abundance compounds or sequences. Since the biological relevance of a compound does not correlate with abundance, it is very important to scale the features in a data set before performing multivariate analyses. This is always a necessary step, but becomes especially important when combining metabolomics data (which may have values into the millions associated with a given compound) with sequencing data (where an OTU table may show proportions of sequences, by definition < 1). For a more complete description of these methods and their application using the online software MetaboAnalyst, the reader is referred to an excellent recent tutorial by Xia and Wishart (2016).

1.5 FATTY ACIDS

The group of compounds known as fatty acids is tremendously diverse, representing a variety of chemicals and physiologically relevant compounds. Chemically, fatty acids are defined by some as “compounds synthesized in nature via condensation of malonyl coenzyme A units by a fatty acid synthase complex” (Christie 2013). More loosely, fatty acids can be described as a carboxylic acid with an aliphatic chain. In many cases, they are grouped with sterols and some other groups of hydrophobic compounds and simply called “lipids”, but for the purpose of this

work, a much smaller subset of these compounds will be explored, but first their context within the realm of lipids should be mentioned.

Among the simplest lipids are triacylglycerols (TAGs), also known in nutrition and medicine by the older term triglycerides. TAGs consist of a glycerol backbone, with a fatty acid esterified to each of the three hydroxyl groups. The specific fatty acid at each position of glycerol can vary in terms of length (number of carbons), units of unsaturation, and type of unsaturated bonds (*cis* or *trans*). TAGs were mentioned previously in terms of dietary intake and absorption of lipids, where lipase is required to hydrolyze them in the GI lumen for absorption by enterocytes and subsequent reassembly and packaging into chylomicrons for transport within the cardiovascular system. TAGs functionally are primarily considered an energy source, including storage in cells, but their component fatty acids are multifunctional.

Similar in structure to TAGs are the phospholipids. These molecules also usually have a glycerol backbone, and two of the three hydroxyl groups of the glycerol are esterified to fatty acids: typically a saturated fatty acid at carbon 1 (C1) and an unsaturated fatty acid at 2. The C3 of glycerol, however, is bound to a phosphate group, which in turn can be bound to one of several small molecules such as choline, serine, or ethanolamine. The phosphate group is extremely hydrophilic compared to the fatty acid tails, creating the amphiphilic character of the phospholipid that permits self-assembly into micelles, liposomes, or bilayers, and ultimately provides for the general character of cell membranes. When the phosphate group is modified, additional chemical properties become accessible (such as the emulsifying ability of lecithin, which is primarily phosphatidylcholine but can be a mix of phospholipids). Another important and structurally similar group is the sphingolipids. In these molecules, a sphingosine backbone plays a role similar to the glycerol backbone of the previously described glycerophospholipids, providing sites for a fatty acid and phosphate group to bond. Again, the identity of the specific fatty acid residue has

important consequences for the final properties of the molecule. These compounds are very important in cell signaling pathways, and include ceramides, sphingomyelins, cerebrosides, and gangliosides, depending on the substituents on the sphingosine backbone.

The genetic regulation of lipid metabolism is quite complicated. The regulation of *de novo* lipogenesis is thought to involve the liver X receptors (LXRα and LXRβ), which are ligand-activated nuclear receptors that particularly respond to cholesterol and oxysterols (Calkin and Tontonoz 2012). Also critical in lipid homeostasis are peroxisome proliferator-activated receptors (PPARs), which are also nuclear receptors, and bind unsaturated fatty acids and other lipids (Kidani and Bensinger 2012). Both PPAR and LXR form a heterodimer with retinoid X receptor (RXR), requiring their endogenous ligand to bind at the same time as 9-*cis*-retinoic acid (or other RXR ligand) to activate transcription of target genes. This is mentioned because farnesoid X receptor (FXR), which is a receptor for bile acids, also forms a heterodimer with RXR, and also requires 9-*cis*-retinoic acid to bind RXR while bile acids are bound to the FXR ligand to activate transcription of FXR target genes. In a gene expression study, FXR (also known as NR1H4) was significantly downregulated, exhibiting a more than 2-fold decrease in expression in duodenal biopsies from dogs with chronic enteropathy relative to healthy control dogs (Wilke et al. 2012). Interestingly, in the same data set NR1H2 (which is LXRβ) was very slightly, but significantly, upregulated (1.17-fold change, q=0.007), and PPARγ, a specific member of the PPAR family with a wide range of effects, was significantly upregulated (4.02-fold change, q=0.001). The important take-home message from this is that metabolism of fatty acids, sterols, and bile acids do not occur independently, and collectively these metabolites interact with nuclear receptors that exert tremendous influence over host metabolism of lipids and carbohydrates, potentially playing a significant role in the pathogenesis of chronic enteropathy and/or IBD in dogs.

Returning to the diversity of fatty acids that can be components of phospholipids, sphingolipids, TAGs, or free fatty acids, a more basic understanding of the biosynthesis and modification of fatty acids is warranted. A descriptive schematic, adapted from the text Lipid Biochemistry (Gurr et al. 2002), is shown in Figure 2.

Among some key features shown in Figure 2 is the fact that long-chain fatty acids are "built" from two-carbon units, and the LCFAs are an important branching point for subsequent conversion to unsaturated fatty acids via desaturases, and VLCFAs via elongases. In other words, palmitic and stearic acids (saturated fatty acids with carbon chain lengths of 16 and 18, respectively) may be considered the precursors for a tremendous library of fatty acids accessible through all the possible variations. Short chain fatty acids (SCFAs) are generally less than eight carbons in length, while medium chain fatty acids (MCFAs) are eight to fourteen carbons. LCFAs

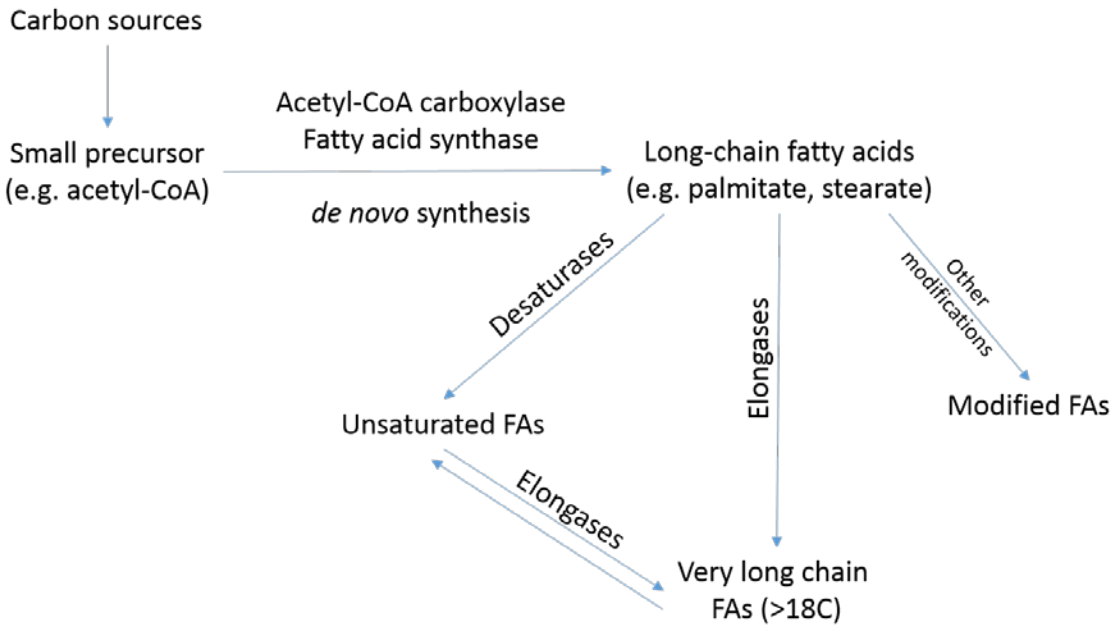

Figure 2. Schematic showing *de novo* biosynthesis and basic transformations leading to the generation of LCFAs, VLCFAs, unsaturated FAs, and other modified FAs Redrawn from Gurr et al. (2002).

are longer than fourteen carbons, with inconsistent distinction given to those longer than 18 carbons as VLCFAs. It is important to note that these divisions are not entirely arbitrary; the chemical properties associated with different lengths of aliphatic chains have biological consequences. Units of unsaturation introduce three types of variability: the number of unsaturated bonds, their location(s), and their conformation (*cis* or *trans*). Regarding conformation, the important point is that a *cis* bond introduces a kink in the carbon chain, while a *trans* bond does not, and this has the effect of lowering the melting point by introduction of disorder – and in a phospholipid bilayer, the kinks help maintain membrane fluidity. The location of the unsaturated bonds is usually specified in relation to the terminal carbon of the aliphatic chain (the methyl group) rather than the carboxyl end, as this better conveys the chemical relationship between two fatty acids. For example, erucic acid and nervonic acid are 22 and 24 carbons in length, respectively, and both have one unit of unsaturation that is 9 carbons from the methyl end. Their shorthand notations are 22:1(*n*-9) and 24:1(*n*-9) respectively, and erucic acid is the precursor to nervonic acid, with the former undergoing an enzymatically driven elongation step (Bourre et al. 1976). Arachidonic acid, consisting of a twenty-carbon chain with four units of unsaturation, has the notation 20:4(*n*-6). Arachidonic acid is a representative of an important classification of fatty acids, the polyunsaturated fatty acids (PUFAs), and is also a key player in the ongoing debate over the importance of the ratio of dietary omega-6 to omega-3 fatty acids (Simopoulos 2002) and subsequent formation of prostaglandins, thromboxanes, and prostacyclins. A wide range of PUFAs are accessible by modification of oleic acid – 18:1(*n*-9), linoleic acid – 18:2(*n*-6), and linolenic acid – 18:3(*n*-3). Fatty acids of the omega-9 series can be synthesized endogenously, while omega-3 and -6 fatty acids start with dietary intake of linoleic and linolenic acids (also referred to as essential fatty acids, EFAs). The question may arise whether unsaturated fatty acids can be restored to saturated fatty acids, and it is worth mentioning that this enzymatic transformation is quite rare

in nature. However, there are ruminal microorganisms (e.g., *Butyrivibrio fibrisolvens*) that can hydrogenate PUFAs to stearic acid (Gurr et al. 2002). This accounts at least in part for the particularly high stearic acid content of beef and dairy fat.

Just as important as biosynthesis of fatty acids is the degradation of fatty acids. As mentioned, dietary TAGs are largely considered a source of energy, but accessing the energy requires breaking of bonds. For a complete discussion of the underlying biochemistry, the reader is referred to the text Lipid Biochemistry (Gurr et al. 2002). In brief, there are three main forms of oxidation, creatively named α, β, and ω. While mitochondria have been historically credited as the "power house" of the eukaryotic cell, VLCFAs must first be oxidized to shorter fatty acid products in order to be accessible to mitochondrial β-oxidation, and this occurs in the peroxisome. Both organelles primarily utilize β-oxidation for fatty acid degradation, and β-oxidation is the most common pathway for fatty acid degradation in mammals. In β-oxidation, the carbon chain is cleaved two carbons at a time, releasing acetyl-CoA into the tricarboxylic acid (TCA) cycle. The rate of β-oxidation is affected by the availability of fatty acids and the accumulation of β-oxidation products. In contrast, α-oxidation occurs only in the peroxisome. This pathway is particularly important in plants, where branched fatty acids are common and structurally cannot undergo β-oxidation, and also can be used to install additional hydroxyl groups on fatty acids. ω-Oxidation generates dicarboxylic acid products from saturated fatty acids, where the terminal methyl group is converted to a second carboxylic acid. Between the requirements of location within the cell and activities of multiple enzymes, these reactions are under many levels of control to maintain homeostasis.

The main focus on fatty acids has been their utility for energy and as a component of larger molecules such as TAGs and phospholipids. Although the variability of the fatty acid composition of phospholipids has been mentioned in terms of affecting structural properties of the phospholipid

bilayer in cell membranes, there is a particularly interesting example of this related to the microbiota. Saito et al. (2014) showed that *Enterococcus faecalis* can incorporate exogenous fatty acids into their membrane from the growth medium, and in doing so, could withstand a wider range of known membrane stressors, including high concentrations of bile salts and the antibiotic daptomycin. Another facet of their research showed that the fatty acid composition of the membrane also changed from the exponential growth phase to the stationary phase, also observed in other Gram-positive bacteria. Therefore, the composition of fatty acids in the lumen of the GI tract in animals may have a direct connection to the physiology of the resident microbiota.

Another physiological function of fatty acids not yet mentioned is related to detoxification in cases of excess cholesterol. Although cholesterol is a very important compound, as will be described in the next section, too much cholesterol presents a problem for cells, and the excess cholesterol must be excreted via the bile. The esters of cholesterol have less of an effect than free cholesterol, so fatty acids may be esterified to cholesterol to mitigate the effect of high cholesterol concentrations while the organism excretes the excess via bile or by conversion to bile acids. The esterification products, *O*-acyl-cholesterol, formed within intestinal cells via the enzyme acyl CoA:cholesterol acyltransferase, ACAT) can then be stored as neutral lipid. This again underscores the importance of fatty acids to sterols, and vice versa, when considering the overall metabolism and homeostasis of lipids.

1.6 STEROLS

Defined by their structure, sterols are a class of compounds with a characteristic four-ring backbone (conventionally identified from left to right, A-D), and a hydroxyl group on the A-ring. The four-ring backbone, also called sterane, is also characteristic of steroids and bile acids, but the hydroxyl group on carbon 3 identifies these as “steroid alcohols” or sterols. Sterols are found in

eukaryotic cells, although plants, animals, and fungi each are associated with a different predominant sterol: β-sitosterol, cholesterol, and ergosterol, respectively. In comparison, prokaryotes are associated with a structurally similar group of molecules, called hopanoids. Hopanoids have a five-ring backbone, and like sterols, are synthesized from squalene. Unlike sterols, the hydrophilic portion of hopanoids is at the equivalent of the alkyl chain on sterol C-17. However, synthesis of hopanoids is anaerobic, while sterol synthesis requires oxygen. This fact is often used to associate evolution of the first eukaryote life with the oxygenation of the atmosphere approximately 2.7 billion years ago. Example structures of these compounds are shown in Figure 3, and the similarity of structure across these varying molecular classes is readily apparent.

The fused ring structure common to all sterols may have one or more units of unsaturation, typically at carbon 5 or 7 (Δ5 or Δ7). The stereochemistry of the hydroxyl group at carbon three may distinguish between two sterols, such as cholesterol and epicholesterol. The alpha configuration projects backwards (axially, relative to the six-carbon ring) and the beta configuration projects forwards (equatorially). Similarly, the stereochemistry of the hydrogen on carbon five has a significant effect on the three-dimensional shape of the molecule overall, where cholestanol (5-alpha) is overall a flatter molecule than the isomer coprostanol (5-beta) since the inversion of the C5 stereocenter forces inversion of the six-carbon ring. A final characteristic of sterols is a variable alkyl chain attached to C17 on the D-ring. While the structural variations may seem small, they may result in significantly different physiological function.

Physiologically, sterols play a critical role in cell structure, making up a large portion of the cell membrane and controlling fluidity and permeability of the membrane. Compared to a simple phospholipid bilayer, the presence of sterols makes the membrane more resilient to variable temperatures (Dufourc 2008). The effects of sterols on membrane characteristics has been studied using model membranes (Vist and Davis 1990). At low temperatures, a model phospholipid bilayer

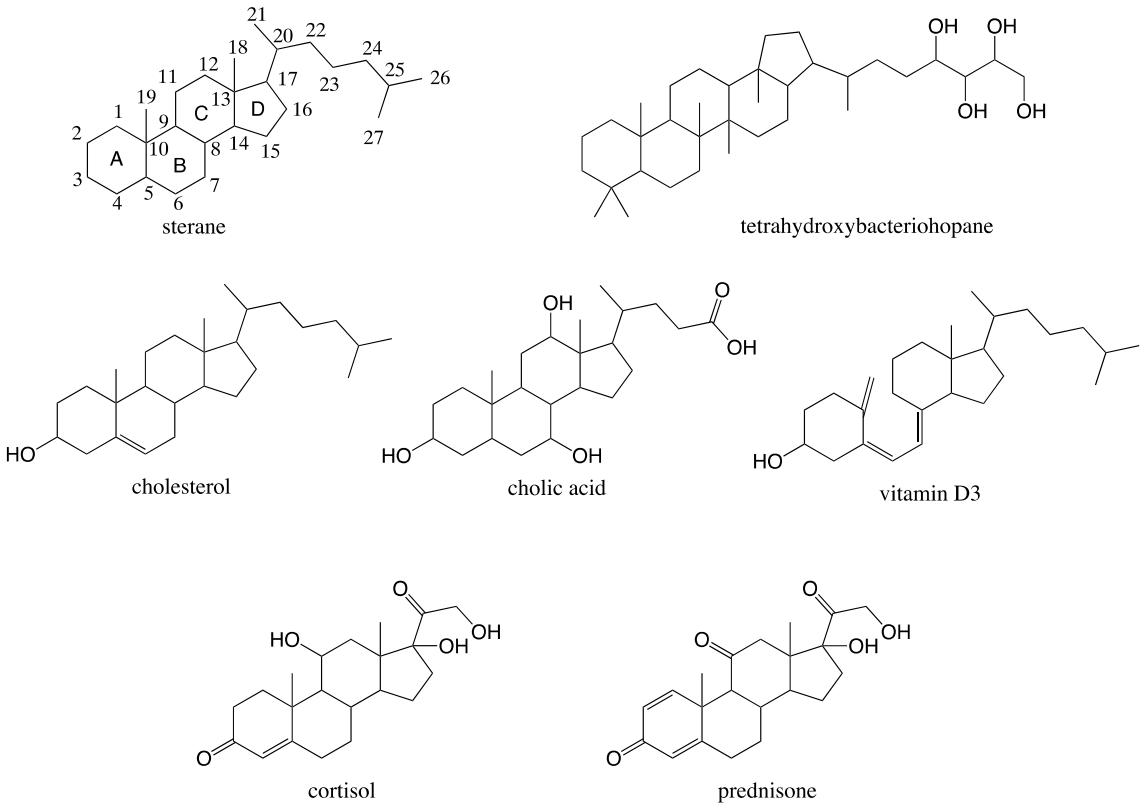

Figure 3. Chemical structures of sterane and related molecules. On sterane (top left), numbering of carbons and letters on rings identifies features used for all sterols. Cholesterol is representative of sterols; cholic acid is representative of bile acids; vitamin D3 is drawn to show its structural relationship to cholesterol as its precursor; cortisol and prednisone are endogenous and exogenous steroids, respectively. Absolute stereochemistry is not shown for any of the compounds.

forms a solid-ordered (*so*) phase, which is rigid and possesses a high degree of order. With increasing temperatures, the bilayer undergoes a phase transition and enters the liquid-disordered (*ld*) phase, which is analogous to ice melting to form water. The *ld* phase has very little structural integrity, corresponding to virtually unlimited permeability. Importantly, like other phase transitions, the *so*-*ld* transition was shown to occur at a specific temperature, meaning the membrane nearly instantaneously went from impermeable and rigid to completely permeable and flaccid at approximately 25°C. However, when model membranes were prepared with at least 20 mol% cholesterol, a distinct phase was observed, the liquid-ordered (*lo*) phase. Not only did the

lo phase demonstrate the possibility of controlled permeability across the membrane, but the sudden transition between phases at a certain temperature no longer occurred: the cholesterol served to disrupt the tight packing of the phospholipids at low temperatures to create a liquid state rather than solid, and at high temperatures, the cholesterol provided increased stability and cohesion, maintaining order. Therefore, although the membrane characteristics still changed with increasing temperature, the degree of change was smaller and it occurred on a steady slope with variation of physiologically-relevant temperatures. Clearly this temperature tolerance for cell membranes is advantageous, even for animals that possess the ability to thermoregulate or move to find compatible temperatures. Combining the effect of cholesterol incorporation with the fatty acid composition of the phospholipid bilayer, the tunability of membrane dynamics for a cell is tremendously vast. One implication of this was explored by Mason et al. (2007), who showed that the sterol composition confers protection to cell membranes when challenged with antimicrobial peptides known to target membrane integrity. Interestingly, they also showed that ergosterol, the principal sterol of fungi, conferred less protection than cholesterol, but demonstrated an intermediate effect compared to membranes lacking sterols.

Also in the cell membrane, cholesterol is a key component in “lipid rafts”, which are important for assembling cell signaling machinery on the cell surface (Lingwood and Simons 2010). Cholesterol is the precursor to bioactive molecules such as vitamin D, hormones (including cortisol), and bile acids. Cholesterol is a constituent of all lipoproteins as a mechanism to get cholesterol to all tissues in the body, as described in Section 1.1. The liver synthesizes cholesterol *de novo*, but much of the body’s need for cholesterol is met by dietary intake and every cell is capable of synthesizing cholesterol, which arguably demonstrates that cholesterol is as important as glucose for cell survival. An interesting tissue variation is the brain, where the blood-brain

barrier prevents importation of cholesterol via lipoproteins, so all cholesterol in the brain must be synthesized *de novo*. Fortunately, the turnover of cholesterol in this tissue is also very slow.

In spite of the necessity of cholesterol for homeostasis, it is detrimental in excess. The primary method for elimination of cholesterol from the total body pool is via conversion to bile acids, which are subsequently excreted into the gastrointestinal tract. The toxicity of excess cholesterol can be mitigated by forming cholesteryl esters – typically phenolic acids or long chain fatty acids are enzymatically esterified at the sterol C3 hydroxyl group. Interestingly, Wilke et al. (2012) showed by microarray analysis that duodenal biopsies of dogs with IBD (relative to healthy dogs) exhibited a 1.6-fold increased expression of cholesterol 7-alpha-hydroxylase, which is the rate-limiting step of conversion of cholesterol to bile acids. This may suggest that a component of the pathogenesis of IBD includes aberration in cholesterol homeostasis and warrants further investigation.

The physiology of cholesterol has been well studied, yet the relevance of other sterols to mammalian health is poorly understood, though an active area of research. Due to consumption of plant material, it can be assumed that phytosterols (plant-origin sterols) enter the gastrointestinal tract. The intestinal absorption of sterols is thought to occur via Niemann-Pick C1-like 1 protein (NPC1L1), and this enzyme does not exclusively absorb cholesterol (though may be less active towards phytosterols). Enterocytes subsequently use transporters ABCG5 and ABCG8 to export phytosterols back into the lumen, and mutations in these transporters in humans are associated with sitosterolemia due to the inability to eliminate sitosterol, the most common phytosterol. In a mouse model that lacks ABCG5 and ABCG8, blood and tissues accumulated sitosterol and campesterol. However, a triple-knock-out mouse model that additionally lacks NPC1L1 did not develop sitosterolemia, suggesting that NPC1L1 is responsible for absorption of phytosterols and the G5/G8 transporters are responsible for selectively expelling non-cholesterol sterols. Based on

experiments in mice, NPC1L1 expression is indirectly decreased by LXR activation, with a concurrent increase in G5 and G8 (Calkin and Tontonoz 2012). As mentioned previously, LXR activation is also tied to *de novo* biosynthesis of fatty acids.

While the function of phytosterols in mammalian cells is unclear, there is evidence that phytosterols are not entirely inert. F. Xu et al. (2005) demonstrated that a mutant cell line with minimal ability to synthesize cholesterol incorporated phytosterols into the cells >90% after 60 days in culture, but also that a small amount of cholesterol was required for continued growth, concluding that phytosterols can substitute for cholesterol as a structural component in the plasma membrane but cannot perform all necessary functions of cholesterol. Given that sterols play an important role in membrane stability, it is conceivable that this incorporation of noncholesterol sterols in the membrane may also impact membrane stability. Some evidence for this *in vivo* was demonstrated by showing that a phytosterol-supplemented diet resulted in decreased severity of disease in a murine model of colitis (Aldini et al. 2014). However, phytosterols are also appreciated as antioxidants, so the mechanism by which the phytosterols exerted the perceived preventative effect is uncertain. In serum, phytosterols may have more negative effects. In pediatric total parenteral nutrition where intravenous lipid emulsions derived from soy lecithin contain relatively high concentrations of phytosterols, a correlation to cholestatic liver disease was observed (Clayton et al. 1998). By comparison to intravenous lipid administered to adults, it was hypothesized that pediatric patients lacked sufficient phytosterol clearance capacity, ultimately affecting function of transporters and resulting in a cascade of dysfunction. Nonetheless, oral administration of phytosterols is still considered beneficial for people with hypercholesterolemia, even as adjunct treatment to statins, with the proposed mechanism being competitive interference of NPC1L1 activity towards cholesterol, thus simply decreasing gastrointestinal absorption (X. Lin et al. 2011).

The physiology of sterols in the context of the microbiota is also relevant, and introduces an interesting host species variation. In humans, the fate of a variable portion of unabsorbed cholesterol in the GI tract is microbial conversion to coprostanol, where the Δ5 bond of cholesterol is hydrogenated (Veiga et al. 2005). A specific strain of *Bacteroides* has been isolated from an individual human and has been shown to convert cholesterol to coprostanol *in vitro*, but the specific strain was not ubiquitous among other individuals known to have a large capacity for reducing cholesterol (Gerard et al. 2007). Other isolated cholesterol-reducing bacteria from non-human animals have been from the genus *Eubacterium* (Gerard 2013). In comparison to humans, the dog is considered in general to be poor at converting cholesterol to coprostanol, to the extent that the sterol composition of fecal pollution in water can be sourced to different animals (Leeming et al. 1996). An interesting study by Antharam et al. (2016) used an integrative approach to combine targeted metabolomics with 16S rRNA sequencing data for fecal samples from humans with *Clostridium difficile* infection (n=7) versus healthy controls (n=6). Although their sample size was small and further subdivided by history of antibiotics usage, they did identify two OTUs with a significant negative correlation to coprostanol and 63 OTUs with a positive correlation. The majority of OTUs positively correlated with coprostanol concentration were taxonomically classified in the order Clostridiales, which has repeatedly been a bacterial group of interest in health and disease in both humans and dogs. A broader and more extensive computational search for relationships between the microbiota and lipid metabolism was performed by Fu et al. (2015), who showed that a model that takes the microbiota into consideration performed better than one that did not when examining risk factors for cardiovascular disease. Neither *C. difficile* nor atherosclerotic cardiovascular disease are as concerning for the canine patient population as for humans (a notable exception is a research colony of cholesterol-sensitive dogs, (Starr 2007)). However, this motif of gastrointestinal protection associated with the microbiota and a correlation

to lipid metabolism may exist among dogs as well. It is clear that the physiology of sterols is quite complicated, and that the role of cholesterol versus other sterols in the dog still requires much investigation.

1.7 HYPOTHESES AND SPECIFIC OBJECTIVES

Though not reviewed in this work, there are several potential biomarkers for IBD in dogs. The specific objectives of this study are not to replace those biomarkers, nor to use molecular techniques to distinguish between diseases that clinically are easily distinguished (i.e., AHDS, IBD, and EPI). As is often joked, clinicians do not need a test to determine whether a dog has diarrhea. Rather, in spite of many ongoing advances in our comprehension of gastrointestinal physiology in health and disease, we are still deficient in a complete understanding of the underlying biochemistry and etiopathophysiology of gastrointestinal diseases. If we attained that complete understanding, decisions of how to diagnose, treat, and track response to therapy would become much less controversial. Thus the objective of this study is to add to the general body of knowledge about canine gastrointestinal physiology in a broad and descriptive sense.

The hypotheses of this project are that

1. The canine gastrointestinal metabolome will be altered in gastrointestinal disease, and
2. These differences in metabolites are associated with changes in the gastrointestinal microbiome.

The specific aims of this study are:

1. to characterize the healthy canine microbiota and metabolome at multiple sites along the gastrointestinal tract
2. to employ untargeted metabolomics approaches on fecal samples to broadly explore potential alterations between dogs with chronic enteropathy and healthy control dogs,

using that information to identify metabolites that may elucidate gastrointestinal physiology and disease pathogenesis

3. to develop and validate a quantitative assay for a subset of relevant metabolites
4. to apply the assay to a broader range of samples from diseased dogs to facilitate additional hypothesis generation and eventually further our understanding of canine gastrointestinal physiology and pathophysiology.

2. VARIATION OF THE MICROBIOTA AND METABOLOME ALONG THE HEALTHY CANINE GASTROINTESTINAL TRACT*

OVERVIEW

The fecal microbiota is relevant to the health and disease of many species. The importance of the fecal metabolome has more recently been appreciated, but our knowledge of the microbiota and metabolome at other sites along the gastrointestinal tract remains deficient. Therefore, we aimed to analyze the gastrointestinal microbiota and metabolome of healthy domestic dogs at four anatomical sites.

Samples of the duodenal, ileal, colonic, and rectal contents were collected from six adult dogs after humane euthanasia for an unrelated study. The microbiota was characterized using Illumina sequencing of 16S rRNA genes. The metabolome was characterized by mass spectrometry-based methods.

Prevalent phyla throughout the samples were Proteobacteria, Firmicutes, Fusobacteria, and Bacteroidetes, consistent with previous findings in dogs and other species. A total of 530 unique metabolites were detected; 199 of these were identified as previously named compounds, but 141 of them had at least one significantly different site-pair comparison. Noteworthy examples include relative concentrations of amino acids, which decreased from the small to large intestine; pyruvate, which peaked in the ileum; and several phenol-containing carboxylic acid compounds that increased in the large intestine.

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In conclusion, the microbiota and metabolome vary significantly at different sites along the canine gastrointestinal tract.

2.1 INTRODUCTION

Eukaryotes co-evolved with prokaryotes over millennia. It is not surprising that coevolution of the gut microbiota with mammalian host species has resulted in commensalism and codependence to maintain homeostasis. The importance of the microbiota has been documented in humans (Cho and Blaser 2012; Shreiner et al. 2015) and companion animals (Hooda et al. 2012; Suchodolski 2011; Kil and Swanson 2011), yet underlying mechanisms of interaction are poorly understood. Bacteria are known to communicate via compounds called auto-inducers (Papenfort and Bassler 2016). Ismail et al. (2016) showed that mammalian epithelial cells produce a compound that mimics a bacterial auto-inducer, suggesting bidirectional communication via metabolites and potential mechanisms that mediate the relationship between the microbiota and the host. Bacterial metabolic activities are also known to respond to changing availability of substrates. Therefore, when considering the gastrointestinal ecosystem, identifying microbiota present is part of characterization, but the biochemical environment – the metabolome – should also be considered.

Investigations into the gastrointestinal metabolome are still relatively new (Saric et al. 2008; Di Cagno et al. 2011; McHardy et al. 2013). This biochemical environment represents the core of symbiosis between host and microbiota: the host provides a nutrient-rich environment, and the microbiota performs functions and produce metabolites for the host that otherwise would be limited or inaccessible. These bacterial metabolites include short chain fatty acids, which provide nutrition for enterocytes (Macfarlane and Macfarlane 1997; Flint et al. 2012); vitamin K, cobalamin, folate, biotin, and thiamine (Hill 1997; LeBlanc et al. 2013); and enzymes to

dehydroxylate and deconjugate endogenous and exogenous metabolites (Winter and Bokkenheuser 1987). The complete repertoire of metabolic assistance provided by the microbiota is far from fully catalogued. Non-targeted metabolomics, which aims to characterize the chemical environment by broadly analyzing samples to identify molecules present, offers another key to describing the gastrointestinal tract (GIT).

Human diseases are often studied through induction of the disease phenotype in a rodent model. However, the veterinary world offers opportunities to study spontaneously occurring diseases that sometimes parallel the disease in man – often both in phenotype and pathogenesis (Honneffer et al. 2014). This is particularly beneficial when the organ of interest is relatively inaccessible *in vivo*, as are some segments of the GIT. To broaden our understanding of mammalian gastrointestinal physiology, the goal of this study was to sample multiple sites along the canine GIT, confirm findings of previous microbiota studies with high throughput sequencing, and investigate the metabolome in conjunction with the microbiota at each site.

2.2 MATERIALS AND METHODS

2.2.1 Study population and sampling

From a colony of apparently healthy adult hound-type dogs (mean age 3.5 years, range 1-11 years), maintained on a commercial adult canine maintenance diet (24% protein, 20% fat), a random subset of six dogs (2 female, 4 male) were sampled. Subjects were fasted at least 8 hours before being humanely euthanized by intravenous injection of a pentobarbital sodium solution at the College of Veterinary Medicine (Texas A&M University, College Station, TX) in accordance with animal use protocol 2015-0164 for reasons unrelated to this study. Carcasses were held at room temperature for up to 3 hours after death while samples were collected. A ventral midline approach was used to access the GIT. At each site, the serosal surface was cut longitudinally to

expose the contents, which were collected using disposable spatulae with as little disruption to the mucosa as possible while collecting material representative of the entire transverse section. Duodenal contents were collected aborally to the cranial flexure. The ileum was identified by presence of the antimesenteric artery. The colon was identified by location *in situ* and samples were taken from the transverse colon or proximal descending colon. Rectal samples were collected as caudally as possible from within the abdominal cavity. Aliquots of approximately 100 mg were collected and stored at -80°C.

2.2.2 DNA extraction and sequencing

Approximately 100 mg of each sample was used for DNA extraction according to the manufacturer's protocol (PowerSoil®, Mo Bio, Carlsbad, CA, USA). Amplification and sequencing of the V4 variable region 16S rRNA gene was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA). Briefly, samples were barcoded and PCR primers 515F/806R were used in a 28-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, with final elongation at 72°C for 5 minutes. A DNA library was prepared according to the Illumina TruSeq DNA library preparation protocol. Sequencing was performed on a MiSeq (Illumina) following the manufacturer's guidelines. Sequence data were uploaded into the NCBI GenBank database under submission number SRP086847.

2.2.3 Analysis of sequences

Quantitative Insights Into Microbial Ecology (QIIME, v. 1.8) was used for processing and analysis of sequences (Caporaso et al. 2010b). Raw sequence data were de-multiplexed, and low quality reads were filtered using default parameters. Chimeric sequences were detected using

USEARCH (Edgar 2010) and removed prior to further analysis. Sequences were assigned to operational taxonomic units (OTUs) using an open-reference picking protocol in QIIME against the Greengenes database (v. 13.8) filtered at ≥97% similarity (Caporaso et al. 2010a; DeSantis et al. 2006; Q. Wang et al. 2007).

2.2.3.1 Diversity metrics

Without filtering rare sequences, samples were rarefied to an even depth of 2,840 reads. Alpha rarefaction plots, coverage and alpha diversity metrics (Goods coverage, Chao1, Shannon, and Observed Species), and beta diversity (weighted and unweighted UniFrac distance matrices) were determined using QIIME scripts (Lozupone and Knight 2005). PRIMER 7 (PRIMER-E Ltd, Ivybridge, UK) was used to compare beta diversity between sites. Variation in community distributions was visualized with Principal Coordinates Analysis (PCoA) plots based on weighted and unweighted UniFrac distances (Vazquez-Baeza et al. 2013). PCoA was chosen for its aptness to representing the phylogenetic similarity distance matrix.

2.2.3.2 Taxonomic summaries

To simplify taxonomic summaries, OTUs present in three or fewer of the samples (to conservatively minimize OTUs that were not consistently represented in a given GI site) were filtered out of the original unrarefied OTU table. Samples were rarefied to an even depth of 1800 reads per sample and QIIME was used to summarize taxa at all phylogenetic levels. Taxa present in at least four samples (again to minimize representation of rare taxa) were analyzed in JMP (SAS, Durham, NC, USA), treating each dog as a block and testing for significant differences with Kruskal-Wallis tests. *P*-values were adjusted using the Benjamini-Hochberg Step-up method, allowing a False Discovery Rate (FDR) of 0.05. A Dunn post-test was used to calculate corrected *p*-values for the six possible site-pair comparisons (duodenum to ileum, colon, or rectum; ileum to colon or rectum, and colon to rectum).

2.2.3.3 Predicted community functional potential

The original OTU table was filtered against the Greengenes database (v. 13.5) to generate a closed-reference OTU table, then rarefied to an even depth of 2,600 reads per sample. The software PICRUSt (Phylogenetic Investigation of Communities through Reconstruction of Unobserved States) (Langille et al. 2013; Paradis et al. 2004) was used to generate a new OTU table comprised of predicted KEGG Orthologs (KOs; Kyoto Encyclopedia of Genes and Genomes) (Kanehisa and Goto 2000; Kanehisa et al. 2016). KOs were categorized into three hierarchical levels of KEGG pathway categories (L1, L2, and L3). The resultant tables were analyzed in JMP in the same method described for the taxonomic summaries.

To explore individual KOs, the uncategorized OTU table was filtered to exclude KOs present in fewer than six of the samples prior to statistical analysis. This cutoff was chosen to conservatively minimize inclusion of KOs that were not represented in all six subjects. Kruskal-Wallis tests and Benjamini-Hochberg corrections were applied to identify predicted KOs that were significantly altered between the gastrointestinal sites. These KOs were cross-referenced using the KEGG mapping tool (http://www.kegg.jp/kegg/tool/map_pathway1.html) to identify pathways with large numbers of significantly altered KOs for further manual investigation.

Further analysis of individual KOs was driven by a large number of hits for a specific pathway, or exploration of specific themes (e.g., to view expression of specific KOs in the bile acid synthesis pathways) rather than comprehensive analysis across all KOs. Particular KOs of interest were analyzed in JMP with a Dunn post-test to identify the site and significance of alteration.

2.2.4 Metabolomics data acquisition

Fecal samples were stored at -80°C until shipped on dry ice for preparation and analysis by the West Coast Metabolomics Core (University of California, Davis, CA, USA) via gas

chromatography time-of-flight mass spectrometry (GC-TOF-MS) in accordance with published methods (Fiehn et al. 2008). Briefly, 10±0.3 mg sample underwent homogenization and extraction, followed by centrifugation. Dried supernatant was resuspended in methanol/chloroform and internal standards were added, followed by drying and derivatization by methoxyamine hydrochloride and N-methyl-N-trimethylsilyltrifluoroacetamide. A volume of 0.5 µL was injected in splitless mode onto a Restek rtx5Sil-MS column on a temperature-gradient programmed GC (oven 50°C to 330°C at 20°C/min, injector 50°C to 250°C at 12°C/sec) coupled with a Leco Pegasus IV mass spectrometer (scanning 70 spectra/sec from 80-500 Da, -70 eV ionization energy, 1,800 V detector voltage) with helium carrier gas (1 mL/min). Raw data files were processed using ChromaTOF v. 2.32. BinBase algorithm matched spectra to database compounds, and quantification was reported by peak height of an ion at the specific retention index characteristic of the compound across all samples. Peak heights were normalized by average total peak-sums for identified compounds across each sample group. Metabolomics data were uploaded to metabolomicsworkbench.org (submission ST000495).

2.2.5 Analysis of metabolites

The peak height data table was filtered to exclude metabolites of unknown identity and uploaded to MetaboAnalyst 3.0 (Xia Lab, McGill University, Canada) (Xia et al. 2015). The data were log transformed and Pareto scaled before statistical analysis (Xia and Wishart 2011). JMP was used for univariate analysis as described previously.

Multivariate analyses were performed within MetaboAnalyst and included principal components analysis (PCA), partial least squares-dimensional analysis (PLS-DA), and random forest analysis (RFA). Based on ranking of features by variable importance in projection (VIP) from PLS-DA, the top 50 features were used to generate a heatmap to visualize metabolomic variability. Cutoffs based on rank were chosen over a specific significance threshold since

different statistical approaches will yield different absolute scores of significance, but most of the top-ranked features are expected to be consistent. For a heatmap, 50 features subjectively provides the ability to visualize both the trends and variability of the features across samples.

2.2.6 Inter-omics analysis

The L5 (family-level) taxonomic summary was concatenated with the metabolite normalized peak intensity table. This was uploaded to MetaboAnalyst, log-transformed and Pareto-scaled, and the Pearson correlation matrix was calculated.

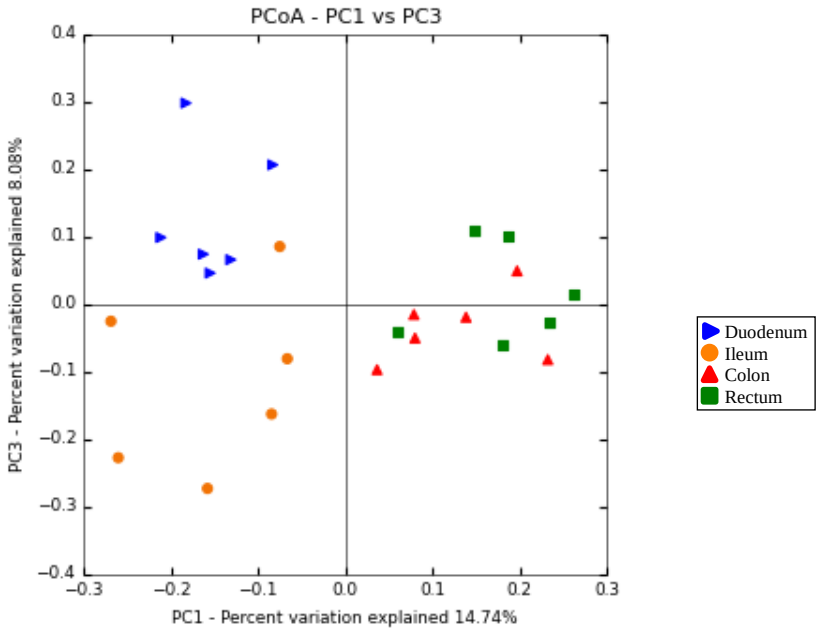


Figure 4. PCoA plot representing beta diversity of microbial communities, based on unweighted UniFrac distance matrices.

2.3 RESULTS

2.3.1 Sequencing data

2.3.1.1 Diversity metrics

Unweighted UniFrac distances demonstrated significantly different beta diversity at all pairwise comparisons (p=0.002) except colon-rectum (p=0.86). Weighted UniFrac identified significantly different beta diversity between duodenum-colon (p=0.0 02), duodenum-rectum (p=0.002), ileum-colon (p=0.035), and ileum-rectum (p=0.006), but not between duodenum-ileum (p=0.31) or colon-rectum (p=0.75). A Principal Coordinates Analysis (PCoA) plot using PCs 1 and 3 is shown in Figure 4, and a chao1 rarefaction curve is shown in Figure 5. Additional diversity metrics and plots are also shown (Table 1 and Figure 6, respectively).

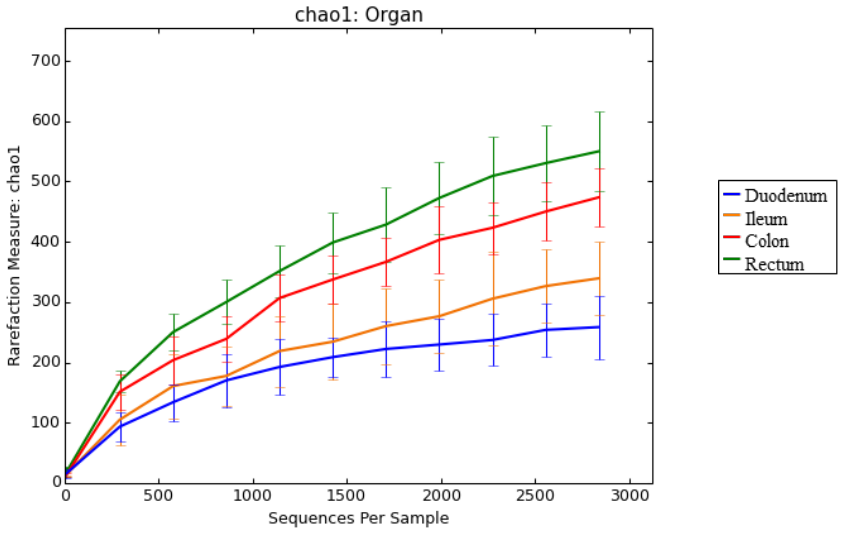


Figure 5. Alpha rarefaction curves of intestinal sites as determined by chao1.

Table 1. Alpha diversity metrics. Superscripts identify which comparisons are significantly different.

Metric	Duodenum		Ileum		Colon		Rectum	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
Chao1	271 [a]	[175-313]	334 [a]	[242-442]	461 [b]	[424-573]	566 [b]	[438-656]
Observed OTUs	157 [a]	[85-210]	145 [a]	[100-238]	212	[199-283]	280 [b]	[232-309]
Shannon	4.7	[2.3-5.7]	4.3	[3.2-4.9]	4.8	[4.4-5.3]	5.5	[4-6.2]
Good's coverage	0.97 [a]	[0.97-0.98]	0.97 [a,b]	[0.95-0.98]	0.96 [b,c]	[0.95-0.96]	0.95 [c]	[0.94-0.96]

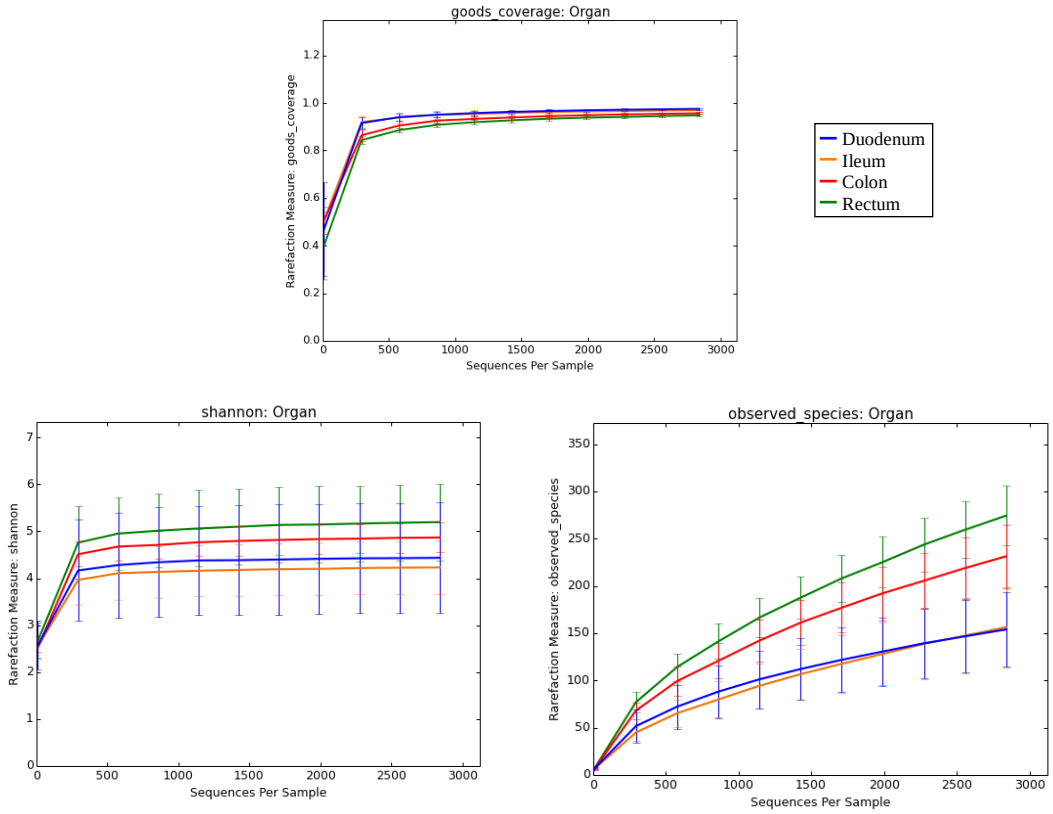


Figure 6. Coverage and alpha diversity plots. These are a visual representation of the sequencing coverage and characteristic diversity of the different GI sites with increasing sequencing depth. The coverage quickly plateaus over 90%, suggesting adequate coverage of the OTUs present. The horizontal line approximated by the Shannon diversity index indicates that the depth of sequencing was adequate. However, the trajectories of the observed species rarefaction curves suggest that deeper sequencing would have yielded additional information, particularly for the large intestinal sites.

2.3.1.2 Taxonomic summary

Predominant phyla sequenced from the four sites were Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes, and Actinobacteria. All except Fusobacteria demonstrated significantly altered abundances across the different sites after the Benjamini-Hochberg correction for multiple comparisons. However, only Actinobacteria and Proteobacteria reached a significance that persisted after the Dunn post-test identified which site-pair comparison was altered (ileum<rectum, q=0.02; and duodenum>rectum, q=0.03, respectively).

Few significant differences were observed at taxonomic levels of class and order. Only the class Coriobacteriia exhibited a difference across sites (ileum<rectum, q=0.008), largely driven by Coriobacteriales, the only order showing a significant difference (ileum<rectum, q=0.008).

Variation of the microbiota was more apparent at the family level. The proportion of Corynebacteriaceae was significantly greater in the duodenum relative to ileum, colon, and rectum (q=0.04, 0.03, and 0.02, respectively). The proportion of Coriobacteriaceae was also significantly altered (ileum<rectum, q=0.008). Bacteroidaceae increased from duodenum to rectum (q=0.02), Prevotellaceae increased from ileum to rectum (q=0.02), and Weeksellaceae was observed only in the duodenum (q=0.049). Lachnospiraceae, Ruminococcaceae, and a poorly defined family also within the order Clostridiales exhibited altered proportions, increasing at distal sites. Altered proportions displayed by families within Proteobacteria included Enterobacteriaceae (ileum>rectum, q=0.02), Helicobacteraceae (duodenum>colon, q=0.02), and Alcaligenaceae (duodenum<rectum, q=0.008).

The median and range of the proportion of OTUs assigned to each taxon are provided in Table 2. The distribution of bacterial orders across organ sites is displayed in Figure 7.

Table 2. Median proportions of OTUs assigned to each taxon. Superscripts indicate which pairwise comparisons are different after Dunn post test.

	Duodenum		Ileum		Colon		Rectum			
Taxon	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
Phylum Actinobacteria	0.02083	[0.00056-0.08444]	0.00083 [a]	[0.00056-0.06]	0.00833	[0.00222-0.10056]	0.01944 [b]	[0.00444-0.09333]	**0.023**	**0.043**
Class Actinobacteria	0.01889	[0-0.08333]	0.00056	[0-0.05722]	0.00111	[0.00056-0.04444]	0.00111	[0-0.05944]	0.108	0.144
Order Actinomycetales	0.01222	[0-0.08167]	0.00028	[0-0.02667]	0.00000	[0-0.00056]	0.00000	[0-0]	**0.009**	0.060
Family Corynebacteriaceae	0.00639 [a]	[0-0.08056]	0.00000 [b]	[0-0.00944]	0.00000 [b]	[0-0.00056]	0.00000 [b]	[0-0]	**0.007**	**0.044**
Corynebacterium	0.00639 [a]	[0-0.08056]	0.00000 [b]	[0-0.00944]	0.00000 [b]	[0-0.00056]	0.00000 [b]	[0-0]	**0.007**	**0.035**
Order Bifidobacteriales	0.00083	[0-0.00722]	0.00028	[0-0.03056]	0.00083	[0.00056-0.04444]	0.00111	[0-0.05944]	0.447	0.498
Family Bifidobacteriaceae	0.00083	[0-0.00722]	0.00028	[0-0.03056]	0.00083	[0.00056-0.04444]	0.00111	[0-0.05944]	0.447	0.447
Bifidobacterium	0.00083	[0-0.00722]	0.00028	[0-0.03056]	0.00083	[0.00056-0.04444]	0.00111	[0-0.05944]	0.447	0.464
Class Coriobacteriia	0.00167	[0.00056-0.01833]	0.00028 [a]	[0-0.00278]	0.00750	[0.00111-0.05611]	0.01833 [b]	[0.00444-0.03389]	**0.002**	**0.022**
Order Coriobacteriales	0.00167	[0.00056-0.01833]	0.00028 [a]	[0-0.00278]	0.00750	[0.00111-0.05611]	0.01833 [b]	[0.00444-0.03389]	**0.002**	**0.034**
Family Coriobacteriaceae	0.00167	[0.00056-0.01833]	0.00028 [a]	[0-0.00278]	0.00750	[0.00111-0.05611]	0.01833 [b]	[0.00444-0.03389]	**0.002**	**0.031**
Collinsella	0.00167	[0.00056-0.01833]	0.00028 [a]	[0-0.00222]	0.00639	[0-0.05611]	0.01556 [b]	[0.00389-0.03389]	**0.003**	**0.028**
Slackia	0.00000	[0-0]	0.00000	[0-0.00056]	0.00000	[0-0.00167]	0.00111	[0-0.00278]	0.073	0.119
Phylum Bacteroidetes	0.00694	[0.00111-0.09722]	0.00167	[0.00056-0.09889]	0.05222	[0-0.12444]	0.12889	[0.00278-0.25667]	**0.034**	**0.043**
Class Bacteroidia	0.00639	[0.00111-0.03667]	0.00167	[0.00056-0.09889]	0.05222	[0-0.12444]	0.12889	[0.00278-0.25667]	**0.016**	0.066
Order Bacteroidales	0.00639	[0.00111-0.03667]	0.00167	[0.00056-0.09889]	0.05222	[0-0.12444]	0.12889	[0.00278-0.25667]	**0.016**	0.062
Family [Paraprevotellaceae]	0.00028	[0-0.01722]	0.00028	[0-0.00278]	0.00083	[0-0.01944]	0.01056	[0-0.01889]	0.115	0.171
[Prevotella]	0.00028	[0-0.01667]	0.00000	[0-0.00111]	0.00056	[0-0.01944]	0.00694	[0-0.01889]	0.131	0.193
CF231	0.00000	[0-0.00056]	0.00000	[0-0.00056]	0.00000	[0-0.00444]	0.00028	[0-0.00444]	0.269	0.322
Family Bacteroidaceae	0.00611 [a]	[0.00056-0.01833]	0.00167	[0-0.095]	0.01528	[0-0.08444]	0.06750 [b]	[0.00222-0.22833]	**0.015**	**0.044**
Bacteroides	0.00611 [a]	[0.00056-0.01833]	0.00167	[0-0.095]	0.01528	[0-0.08444]	0.06750 [b]	[0.00222-0.22833]	**0.015**	**0.035**
Family Prevotellaceae	0.00056	[0-0.01]	0.00000 [a]	[0-0.00167]	0.00250	[0-0.12278]	0.01583 [b]	[0.00056-0.05833]	**0.013**	**0.044**
Prevotella	0.00056	[0-0.01]	0.00000 [a]	[0-0.00167]	0.00250	[0-0.12278]	0.01583 [b]	[0.00056-0.05833]	**0.013**	**0.035**
Family S24-7	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0.00167]	0.00000	[0-0.00167]	0.381	0.392
g__	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0.00167]	0.00000	[0-0.00167]	0.381	0.419
Class Flavobacteriia	0.00111	[0-0.06056]	0.00000	[0-0]	0.00000	[0-0]	0.00000	[0-0]	**0.013**	0.066
Order Flavobacteriales	0.00111	[0-0.06056]	0.00000	[0-0]	0.00000	[0-0]	0.00000	[0-0]	**0.013**	0.060
Family [Weeksellaceae]	0.00111 [a]	[0-0.06056]	0.00000 [b]	[0-0]	0.00000 [b]	[0-0]	0.00000 [b]	[0-0]	**0.013**	**0.044**

Table 2. (Continued.)

Taxon	Duodenum		Ileum		Colon		Rectum			
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
Phylum Firmicutes	0.15500	[0.01167-0.68056]	0.30917	[0.02333-0.93111]	0.66222	[0.53056-0.84389]	0.66083	[0.54056-0.86833]	**0.028**	**0.043**
Class Bacilli	0.07667	[0.00556-0.46056]	0.03472	[0.00222-0.55389]	0.22806	[0.02111-0.48]	0.24833	[0.04722-0.65667]	0.147	0.160
Order Bacillales	0.00139	[0-0.19056]	0.00083	[0-0.12722]	0.00000	[0-0.00056]	0.00028	[0-0.00056]	0.177	0.225
Family Staphylococcaceae	0.00139	[0-0.09222]	0.00083	[0-0.12222]	0.00000	[0-0.00056]	0.00028	[0-0.00056]	0.209	0.263
Staphylococcus	0.00139	[0-0.09222]	0.00083	[0-0.12222]	0.00000	[0-0.00056]	0.00028	[0-0.00056]	0.209	0.267
Order Gemellales	0.00056	[0-0.01444]	0.00000	[0-0.00167]	0.00000	[0-0.00056]	0.00000	[0-0]	0.134	0.181
Family Gemellaceae	0.00056	[0-0.01444]	0.00000	[0-0.00167]	0.00000	[0-0.00056]	0.00000	[0-0]	0.134	0.189
Gemella	0.00056	[0-0.01444]	0.00000	[0-0.00167]	0.00000	[0-0.00056]	0.00000	[0-0]	0.134	0.193
Order Lactobacillales	0.02583	[0.00111-0.18222]	0.01250	[0.00111-0.18111]	0.00583	[0.00333-0.32278]	0.00361	[0.00111-0.32167]	0.498	0.498
Family Aerococcaceae	0.00028	[0-0.035]	0.00139	[0-0.02556]	0.00000	[0-0]	0.00028	[0-0.00056]	0.198	0.259
g__	0.00028	[0-0.035]	0.00139	[0-0.02556]	0.00000	[0-0]	0.00028	[0-0.00056]	0.198	0.260
Family Enterococcaceae	0.00028	[0-0.00056]	0.00056	[0-0.01278]	0.00000	[0-0.00111]	0.00000	[0-0.01778]	0.169	0.229
Enterococcus	0.00028	[0-0.00056]	0.00056	[0-0.01222]	0.00000	[0-0.00111]	0.00000	[0-0.01778]	0.169	0.231
Family Lactobacillaceae	0.00583	[0.00056-0.02611]	0.00111	[0.00111-0.09444]	0.00306	[0.00222-0.32222]	0.00139	[0.00056-0.32111]	0.256	0.290
Lactobacillus	0.00583	[0.00056-0.02611]	0.00111	[0.00111-0.09444]	0.00306	[0.00222-0.32222]	0.00139	[0.00056-0.32111]	0.256	0.313
Family Streptococcaceae	0.00833	[0-0.17]	0.00250	[0-0.05833]	0.00083	[0-0.00722]	0.00056	[0-0.07]	0.254	0.290
Streptococcus	0.00833	[0-0.17]	0.00250	[0-0.05833]	0.00083	[0-0.00722]	0.00056	[0-0.07]	0.254	0.313
Order Turicibacterales	0.01861	[0.00333-0.13389]	0.00806	[0.00111-0.31056]	0.18556	[0.01611-0.40389]	0.24556	[0.04556-0.54056]	**0.030**	0.081
Family Turicibacteraceae	0.01861	[0.00333-0.13389]	0.00806	[0.00111-0.31056]	0.18556	[0.01611-0.40389]	0.24556	[0.04556-0.54056]	**0.030**	0.067
Turicibacter	0.01861	[0.00333-0.13389]	0.00806	[0.00111-0.31056]	0.18556	[0.01611-0.40389]	0.24556	[0.04556-0.54056]	**0.030**	0.056
Class Clostridia	0.07722	[0.00611-0.21833]	0.17139	[0.02111-0.58611]	0.38444	[0.24444-0.58222]	0.40222	[0.16944-0.565]	**0.034**	0.068
Order Clostridiales	0.07722	[0.00611-0.21833]	0.17139	[0.02111-0.58611]	0.38444	[0.24444-0.58222]	0.40222	[0.16944-0.565]	**0.034**	0.081
f__	0.00028	[0-0.00444]	0.00000 [a]	[0-0.00056]	0.00472 [b]	[0.00278-0.00667]	0.00444 [b]	[0.00167-0.005]	**0.003**	**0.037**
g__	0.00028	[0-0.00444]	0.00000 [a]	[0-0.00056]	0.00472 [b]	[0.00278-0.00667]	0.00444 [b]	[0.00167-0.005]	**0.003**	**0.030**
Family Clostridiaceae	0.03028	[0.00333-0.08556]	0.04000	[0.00333-0.225]	0.16889	[0.01278-0.40889]	0.13722	[0.02333-0.18167]	0.095	0.153
g__	0.00111 [a]	[0-0.00333]	0.00250	[0-0.01111]	0.01333 [b]	[0.00278-0.01889]	0.01389 [b]	[0.00167-0.02111]	**0.006**	**0.035**
Candidatus Arthromitus	0.00083	[0-0.00667]	0.00167	[0-0.15722]	0.00028	[0-0.00056]	0.00000	[0-0.00056]	0.108	0.165
Clostridium	0.00056	[0-0.00167]	0.00056 [a]	[0-0.00222]	0.00361 [b]	[0.00167-0.00444]	0.00111	[0-0.01111]	**0.012**	**0.035**
SMB53	0.00056 [a]	[0-0.00333]	0.00056 [a]	[0-0.00222]	0.00972 [b]	[0.00056-0.01389]	0.00528	[0-0.00722]	**0.011**	**0.035**
Other	0.02417	[0.00222-0.07944]	0.01722 [a]	[0.00111-0.05667]	0.13722 [b]	[0.005-0.37278]	0.11333	[0.00944-0.15667]	**0.009**	**0.035**

Table 2. (Continued.)

Taxon	Duodenum		Ileum		Colon		Rectum		*p*-value	*q*-value
	Median	Range	Median	Range	Median	Range	Median	Range		
Family Lachnospiraceae	0.01000	[0.00111-0.11056]	0.00361 [a]	[0.00167-0.00778]	0.12833 [b]	[0.06278-0.21056]	0.11694 [b]	[0.075-0.30333]	**0.001**	**0.031**
g__	0.00111 [a,b]	[0-0.01]	0.00028 [a]	[0-0.00167]	0.01278 [b,c]	[0.00556-0.01833]	0.01444 [c]	[0.00722-0.01833]	**0.001**	**0.021**
[Ruminococcus]	0.00222 [a]	[0-0.00556]	0.00083 [a]	[0.00056-0.00111]	0.01361 [b]	[0.00389-0.04833]	0.01667 [b]	[0.01167-0.03167]	**0.001**	**0.021**
Blautia	0.00333	[0-0.04]	0.00056 [a]	[0-0.00333]	0.05167 [b]	[0.01833-0.12222]	0.06361 [b]	[0.02611-0.17778]	**0.002**	**0.021**
Coprococcus	0.00028	[0-0.00056]	0.00000 [a]	[0-0]	0.00111 [b]	[0.00056-0.00444]	0.00194 [b]	[0-0.005]	**0.004**	**0.033**
Dorea	0.00278	[0-0.05167]	0.00111 [a]	[0-0.00222]	0.02250 [b]	[0.01056-0.06722]	0.01139	[0.00556-0.03944]	**0.012**	**0.035**
Other	0.00056	[0-0.00278]	0.00028 [a]	[0-0.00111]	0.00389 [b]	[0.00056-0.01722]	0.00417	[0.00056-0.04333]	**0.006**	**0.035**
Family Peptococcaceae	0.00028	[0-0.00222]	0.00000	[0-0.00056]	0.00306	[0-0.03111]	0.00111	[0-0.03111]	**0.022**	0.054
Peptococcus	0.00028	[0-0.00222]	0.00000	[0-0.00056]	0.00306	[0-0.03111]	0.00111	[0-0.03111]	**0.022**	**0.045**
Family Peptostreptococcaceae	0.00778	[0-0.01333]	0.11139	[0.00111-0.30722]	0.01917	[0.00278-0.05944]	0.01139	[0.00333-0.05333]	0.075	0.134
g__	0.00444	[0-0.01278]	0.06250	[0.00056-0.21167]	0.00389	[0.00222-0.05444]	0.01056	[0.00111-0.05111]	0.065	0.116
Other	0.00194	[0-0.00611]	0.01778	[0.00056-0.13333]	0.00472	[0.00056-0.05222]	0.00167	[0.00056-0.00667]	0.069	0.118
Family Ruminococcaceae	0.00056	[0-0.00389]	0.00028 [a]	[0-0.00056]	0.00000	[0-0.01389]	0.01083 [b]	[0.00167-0.06722]	**0.014**	**0.044**
g__	0.00000	[0-0.00333]	0.00000 [a]	[0-0.00056]	0.00000	[0-0.00611]	0.00417 [b]	[0.00056-0.01778]	**0.025**	**0.050**
Faecalibacterium	0.00056	[0-0.00056]	0.00000 [a]	[0-0.00056]	0.00000	[0-0.00722]	0.00639 [b]	[0-0.04833]	**0.015**	**0.035**
Family Veillonellaceae	0.00361	[0.00056-0.01778]	0.00111	[0-0.00944]	0.03583	[0.00056-0.26389]	0.04583	[0.005-0.09778]	**0.015**	**0.044**
Megamonas	0.00139	[0.00056-0.00333]	0.00083	[0-0.00556]	0.00889	[0.00056-0.26111]	0.02556	[0.00167-0.06111]	**0.015**	**0.035**
Megasphaera	0.00000	[0-0.01611]	0.00000	[0-0.00056]	0.00056	[0-0.04]	0.00000	[0-0.03889]	0.293	0.336
Phascolarctobacterium	0.00139 [a]	[0-0.00333]	0.00000 [a]	[0-0.00667]	0.00250	[0-0.02889]	0.01500 [b]	[0.00056-0.03611]	**0.014**	**0.035**
Other	0.00111	[0-0.11056]	0.03250	[0.00111-0.13333]	0.01083	[0.00333-0.01389]	0.00972	[0.005-0.01556]	0.355	0.377
Other	0.00111	[0-0.11056]	0.03250	[0.00111-0.13333]	0.01083	[0.00333-0.01389]	0.00972	[0.005-0.01556]	0.355	0.398
Class Erysipelotrichi	0.00139	[0-0.00222]	0.00028	[0-0.09889]	0.01611	[0.00167-0.05556]	0.01028	[0.005-0.02389]	0.086	0.129
Order Erysipelotrichales	0.00139	[0-0.00222]	0.00028	[0-0.09889]	0.01611	[0.00167-0.05556]	0.01028	[0.005-0.02389]	0.086	0.136
Family Erysipelotrichaceae	0.00139	[0-0.00222]	0.00028	[0-0.09889]	0.01611	[0.00167-0.05556]	0.01028	[0.005-0.02389]	0.086	0.146
g__	0.00000	[0-0]	0.00000	[0-0]	0.00000	[0-0.00056]	0.00028	[0-0.005]	0.105	0.165
[Eubacterium]	0.00000	[0-0.00056]	0.00000 [a]	[0-0.00056]	0.00111 [b]	[0-0.00556]	0.00056	[0-0.00222]	**0.011**	**0.035**
Allobaculum	0.00111	[0-0.00167]	0.00000	[0-0.09833]	0.00250	[0.00056-0.03389]	0.00278	[0.00111-0.01667]	0.172	0.231
Catenibacterium	0.00000 [a]	[0-0.00056]	0.00000 [a]	[0-0.00056]	0.00361 [b]	[0.00111-0.02333]	0.00528	[0.00167-0.00833]	**0.001**	**0.021**

Table 2. (Continued.)

Taxon	Duodenum		Ileum		Colon		Rectum			
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
Phylum Fusobacteria	0.03306	[0.00389-0.26389]	0.00361	[0.00222-0.32722]	0.12472	[0.00111-0.36111]	0.06333	[0.00889-0.32556]	0.478	0.478
Class Fusobacteriia	0.03306	[0.00389-0.26389]	0.00361	[0.00222-0.32722]	0.12472	[0.00111-0.36111]	0.06333	[0.00889-0.32556]	0.478	0.478
Order Fusobacteriales	0.03306	[0.00389-0.26389]	0.00361	[0.00222-0.32722]	0.12472	[0.00111-0.36111]	0.06333	[0.00889-0.32556]	0.478	0.498
Family Fusobacteriaceae	0.02056	[0.00389-0.04667]	0.00361	[0.00222-0.32722]	0.12472	[0.00111-0.36111]	0.06306	[0.00889-0.32556]	0.242	0.290
Fusobacterium	0.02056	[0.00389-0.04611]	0.00361	[0.00222-0.32444]	0.11694	[0.00111-0.34778]	0.05806	[0.00722-0.31167]	0.282	0.329
Other	0.00000	[0-0.00222]	0.00000 [a]	[0-0.00278]	0.00444 [b]	[0-0.015]	0.00278	[0-0.01389]	**0.007**	**0.035**
Family Leptotrichiaceae	0.00167	[0-0.21722]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0.00056]	**0.022**	0.054
g__	0.00167	[0-0.21722]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0.00056]	**0.022**	**0.045**
Phylum Proteobacteria	0.59139 [a]	[0.14833-0.97778]	0.51389	[0.005-0.90944]	0.06306	[0.00667-0.255]	0.04806 [b]	[0.01278-0.12556]	**0.007**	**0.033**
Class Alphaproteobacteria	0.00306	[0-0.03444]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0]	0.074	0.126
Order Rhizobiales	0.00056	[0-0.03056]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0]	0.074	0.127
Family Methylobacteriaceae	0.00056	[0-0.03056]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0]	0.074	0.134
Methylobacterium	0.00056	[0-0.03056]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0]	0.074	0.119
Class Betaproteobacteria	0.00306	[0.00056-0.07556]	0.00139	[0-0.01333]	0.00306	[0.00056-0.01444]	0.00917	[0.00389-0.02611]	0.123	0.147
Order Burkholderiales	0.00167	[0-0.07556]	0.00139	[0-0.01333]	0.00306	[0.00056-0.01444]	0.00917	[0.00389-0.02611]	0.262	0.311
Family Alcaligenaceae	0.00083 [a]	[0-0.00111]	0.00139	[0-0.01333]	0.00306	[0.00056-0.01444]	0.00917 [b]	[0.00389-0.02611]	**0.011**	**0.044**
Sutterella	0.00083 [a]	[0-0.00111]	0.00139	[0-0.01333]	0.00306	[0.00056-0.01444]	0.00917 [b]	[0.00389-0.02611]	**0.011**	**0.035**
Class Epsilonproteobacteria	0.03667	[0.00056-0.93333]	0.00056	[0-0.00667]	0.00000	[0-0.00056]	0.00056	[0-0.00278]	**0.034**	0.068
Order Campylobacterales	0.03667	[0.00056-0.93333]	0.00056	[0-0.00667]	0.00000	[0-0.00056]	0.00056	[0-0.00278]	**0.034**	0.081
Family Campylobacteraceae	0.00028	[0-0.06889]	0.00000	[0-0.00611]	0.00000	[0-0.00056]	0.00000	[0-0.00056]	0.321	0.352
Campylobacter	0.00000	[0-0.005]	0.00000	[0-0.00556]	0.00000	[0-0.00056]	0.00000	[0-0.00056]	0.591	0.602
Family Helicobacteraceae	0.00194 [a]	[0.00056-0.93278]	0.00056	[0-0.00111]	0.00000 [b]	[0-0]	0.00028	[0-0.00222]	**0.011**	**0.044**
Helicobacter	0.00194 [a]	[0.00056-0.93278]	0.00028	[0-0.00111]	0.00000 [b]	[0-0]	0.00028	[0-0.00222]	**0.010**	**0.035**
Class Gammaproteobacteria	0.27389	[0.00778-0.85389]	0.50306	[0.00389-0.90833]	0.06139	[0.00333-0.24889]	0.03472	[0.00389-0.11667]	**0.034**	0.068
Order Aeromonadales	0.00083	[0-0.00389]	0.00111	[0-0.01]	0.00250	[0.00167-0.09167]	0.01056	[0.00056-0.11111]	0.099	0.145
Family Succinivibrionaceae	0.00083	[0-0.00389]	0.00111	[0-0.01]	0.00250	[0.00167-0.09167]	0.01056	[0.00056-0.11111]	0.099	0.153
g__	0.00000	[0-0]	0.00000	[0-0.00056]	0.00000	[0-0.00056]	0.00000	[0-0.00222]	0.400	0.431
Anaerobiospirillum	0.00083	[0-0.00389]	0.00083	[0-0.00889]	0.00194	[0-0.09167]	0.01028	[0.00056-0.11056]	0.147	0.208
Succinivibrio	0.00000	[0-0]	0.00000	[0-0.00056]	0.00000	[0-0.02167]	0.00000	[0-0.00611]	0.761	0.761

Table 2. (Continued.)

Taxon	Duodenum		Ileum		Colon		Rectum			
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
Order Enterobacteriales	0.11611	[0.00778-0.85]	0.49778	[0.00278-0.90722]	0.00583	[0.00056-0.24667]	0.00361	[0.00056-0.06556]	**0.011**	0.060
Family Enterobacteriaceae	0.11611	[0.00778-0.85]	0.49778 [a]	[0.00278-0.90722]	0.00583	[0.00056-0.24667]	0.00361 [b]	[0.00056-0.06556]	**0.011**	**0.044**
g__	0.10056	[0.00667-0.79667]	0.43750 [a]	[0.00278-0.82]	0.00472	[0.00056-0.21556]	0.00333 [b]	[0-0.05778]	**0.011**	**0.035**
Erwinia	0.00000	[0-0.01833]	0.00000	[0-0.02889]	0.00000	[0-0.00056]	0.00000	[0-0]	0.407	0.431
Other	0.01556	[0.00111-0.08611]	0.05944 [a]	[0-0.11389]	0.00139	[0-0.02778]	0.00056 [b]	[0-0.00778]	**0.018**	**0.040**
Order Pasteurellales	0.00139	[0-0.22611]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0.00056]	**0.039**	0.082
Family Pasteurellaceae	0.00139	[0-0.22611]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0.00056]	**0.039**	0.083
g__	0.00111	[0-0.19833]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0.00056]	**0.039**	0.072
Order Pseudomonadales	0.00167	[0-0.085]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0]	0.074	0.127
Family Pseudomonadaceae	0.00167	[0-0.085]	0.00000	[0-0.00056]	0.00000	[0-0]	0.00000	[0-0]	0.074	0.134

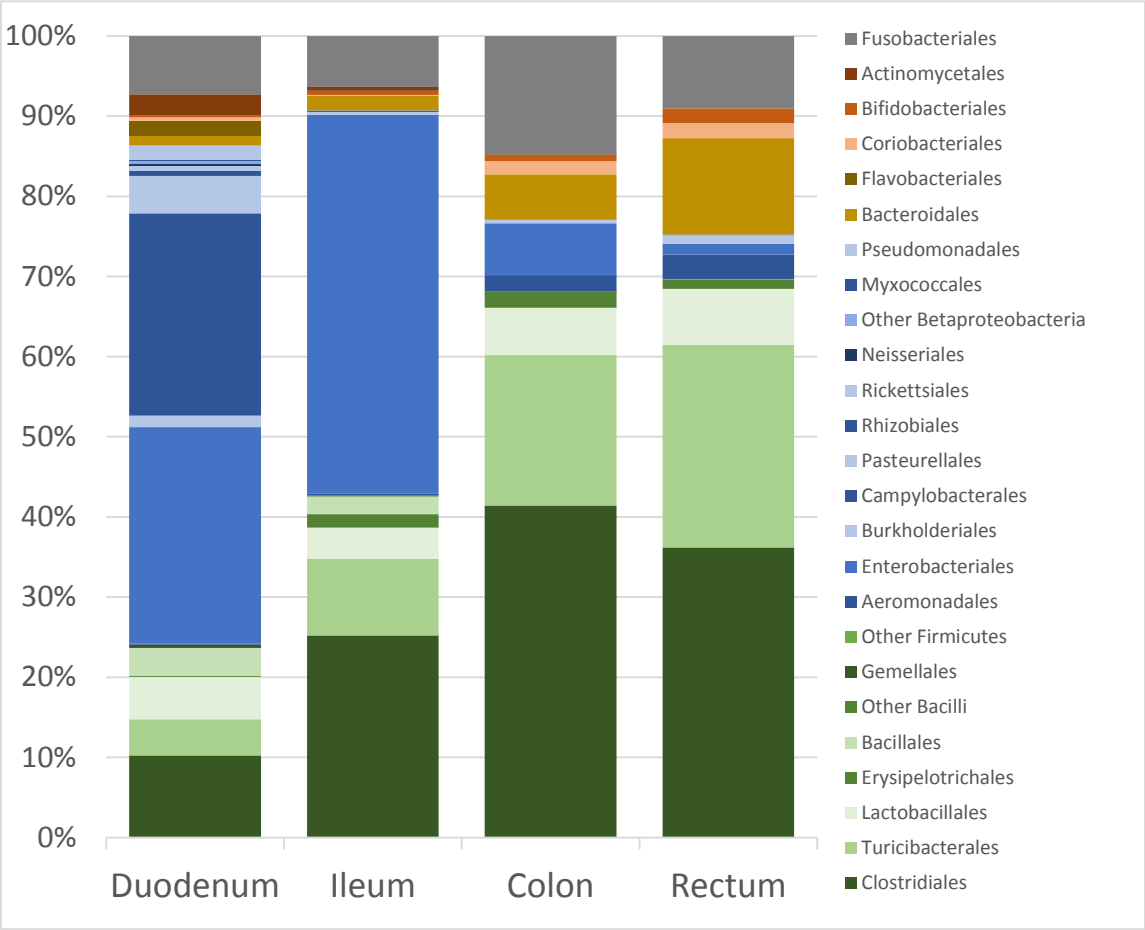


Figure 7. Taxonomic summary of bacterial orders by intestinal segment. Orders within Firmicutes are in shades of green, while orders within Proteobacteria are in shades of blue.

2.3.1.3 PICRUSt analysis results

At the L1 hierarchy, pathway categories Cellular Processes and Human Diseases were predicted to have differential metagenomic potential between sites (both q=0.015). Within Cellular Processes, the difference was largely driven by KOs categorized into pathways of Cell Motility (q=0.027). Within Human Diseases, significance was driven by categories of Cardiovascular Diseases, Infectious Diseases, and Neurodegenerative Diseases (all q=0.027). Other differences at the L2 level were Signal Transduction, Circulatory System, and Excretory System (q=0.027).

There were 85 L3 pathway categories that reached significance. All significant categorized alterations of community functional potential at all hierarchical levels are presented in the Appendix, Table A-1.

Among uncategorized KOs, 4,558 were present in at least six of the 24 samples. Of these, 2,945 exhibited differential abundance in at least one pairwise comparison of sites. Only 1,428 were mapped to existing KEGG pathway maps.

Because the functional potential of the microbiota with regard to bile acid processing would depend on the gastrointestinal site, the KOs categorized into the L3 pathway categories Primary Bile Acid Biosynthesis (KEGG map00120) and Secondary Bile Acid Synthesis (KEGG map00121) were extracted from KEGG. These contained 18 and 8 KOs respectively. Cross-referencing with individual KOs from PICRUSt output, K01796 and K01442 exhibited significant alterations (q=0.031 and 0.017, respectively).

The data for these two KOs are shown in Table 3. K01442 is identified in KEGG as the enzyme choloylglycine hydrolase [EC:3.5.1.24] and is responsible for hydrolytic deconjugation of bile acids. K01796 is identified as orthologous to alpha-methylacyl-CoA racemase [EC:5.1.99.4], an enzyme responsible for inversion of the C25 stereocenter during synthesis of cholate and chenodeoxycholate from cholesterol.

The pathway “Flagellar Assembly” (KEGG map02040) has obvious relevance to the gastrointestinal microbiota and was an altered L3 pathway (q=0.013). Querying KEGG revealed this pathway contains 37 KOs; all were present in the data set, and 34 were among those significantly different at sites in the GIT (Table 4).

Table 3. Abundance of KOs associated with bile acid synthesis. P- and q-values <0.05 are shown in bold; superscripts identify which comparisons are statistically significantly different.

KEGG Ortholog	Duodenum		Ileum		Colon		Rectum			
	Median	Range	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
K01442 E3.5.1.24; choloylglycine hydrolase	182.5 [a]	[26-307]	251.5 [a,b]	[177-389]	465.5 [b,c]	[365-923]	648.5 [c]	[434-933]	**0.001**	**0.017**
K01796 E5.1.99.4, AMACR; alpha-methylacyl-CoA racemase	16.5 [a]	[0-244]	0.5 [b]	[0-5]	4	[0-7]	2.5	[0-6]	**0.016**	**0.031**

Table 4. Abundance of KOs associated with the flagellar assembly pathway. *P*- and *q*-values <0.05 are shown in bold; superscripts identify which comparisons are statistically significantly different.

KEGG Ortholog	Duodenum		Ileum		Colon		Rectum			
	Median	Range	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
K02386 flgA; flagellar basal-body P-ring formation protein FlgA	420.5 [a]	[105-1134]	307.5	[4-456]	3.5 [b]	[1-115]	1.5 [b]	[0-33]	**0.002**	**0.019**
K02387 flgB; flagellar basal-body rod protein FlgB	436 [a]	[266-1149]	477 [a,b]	[256-793]	143 [b,c]	[40-266]	87.5 [c]	[41-125]	**0.001**	**0.017**
K02388 flgC; flagellar basal-body rod protein FlgC	444 [a]	[271-1306]	485.5 [a,b]	[257-796]	144 [b,c]	[40-266]	88 [c]	[41-125]	**0.001**	**0.017**
K02389 flgD; flagellar basal-body rod modification protein FlgD	442.5 [a]	[265-1149]	426 [a,b]	[256-796]	143.5 [b,c]	[36-265]	87 [c]	[37-124]	**0.001**	**0.017**
K02390 flgE; flagellar hook protein FlgE	440.5 [a]	[258-2422]	435.5	[204-621]	79.5 [b]	[29-232]	68 [b]	[33-83]	**0.004**	**0.020**
K02391 flgF; flagellar basal-body rod protein FlgF	120.5	[14-447]	309.5 [a]	[3-464]	2	[1-119]	1.5 [b]	[0-35]	**0.004**	**0.020**
K02392 flgG; flagellar basal-body rod protein FlgG	445.5	[388-2388]	551	[273-1080]	390	[340-545]	387	[209-499]	0.106	0.136
K02393 flgH; flagellar L-ring protein precursor FlgH	433.5 [a]	[168-1135]	318	[4-477]	43 [b]	[5-139]	16 [b]	[10-36]	**0.002**	**0.017**
K02394 flgI; flagellar P-ring protein precursor FlgI	433.5 [a]	[168-1140]	318	[4-477]	43 [b]	[5-139]	16 [b]	[10-36]	**0.002**	**0.017**
K02396 flgK; flagellar hook-associated protein 1 FlgK	433 [a]	[243-1150]	437.5	[229-664]	237.5	[176-289]	201.5 [b]	[155-343]	**0.008**	**0.023**
K02397 flgL; flagellar hook-associated protein 3 FlgL	438.5 [a]	[204-1138]	431.5 [a,b]	[203-620]	67.5 [b,c]	[18-139]	43.5 [c]	[14-63]	**0.001**	**0.017**
K02398 flgM; negative regulator of flagellin synthesis FlgM	139.5	[15-483]	413.5 [a]	[202-636]	77 [b]	[18-155]	47.5 [b]	[13-69]	**0.002**	**0.019**
K02399 flgN; flagella synthesis protein FlgN	106	[10-444]	309.5 [a]	[2-462]	2 [b]	[1-119]	1.5 [b]	[0-35]	**0.005**	**0.020**
K02400 flhA; flagellar biosynthesis protein FlhA	474 [a]	[266-1151]	536 [a]	[256-815]	149.5 [b]	[40-266]	89.5 [b]	[41-126]	**0.001**	**0.017**
K02401 flhB; flagellar biosynthetic protein FlhB	423 [a]	[187-1097]	309.5	[7-460]	69 [b]	[23-155]	37.5 [b]	[23-69]	**0.002**	**0.018**
K02402 flhC; flagellar transcriptional activator FlhC	89	[10-436]	305 [a]	[2-453]	2 [b]	[1-115]	1.5 [b]	[0-33]	**0.005**	**0.020**
K02403 flhD; flagellar transcriptional activator FlhD	89	[10-437]	305 [a]	[2-453]	2 [b]	[1-115]	1.5 [b]	[0-33]	**0.005**	**0.020**

Table 4. (Continued.)

KEGG Ortholog		Duodenum		Ileum		Colon		Rectum			
		Median	Range	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
K02406	fliC; flagellin	636	[407-2319]	598.5	[297-1054]	606.5	[460-729]	532.5	[310-817]	0.409	0.440
K02407	fliD; flagellar hook-associated protein 2	435.5 [a]	[215-1139]	427	[204-618]	175.5 [b]	[131-257]	159 [b]	[137-286]	**0.004**	**0.020**
K02408	fliE; flagellar hook-basal body complex protein FliE	264.5	[146-450]	476.5 [a]	[256-793]	143 [b]	[40-266]	87.5 [b]	[41-125]	**0.000**	**0.017**
K02409	fliF; flagellar M-ring protein FliF	436 [a]	[265-1143]	476 [a,b]	[256-792]	138 [b,c]	[31-260]	81 [c]	[36-119]	**0.001**	**0.017**
K02410	fliG; flagellar motor switch protein FliG	436 [a]	[266-1152]	477 [a,b]	[256-793]	143 [b,c]	[40-266]	87.5 [c]	[41-125]	**0.001**	**0.017**
K02411	fliH; flagellar assembly protein FliH	430.5 [a]	[262-1139]	407 [a,b]	[254-789]	122.5 [b,c]	[28-251]	72.5 [c]	[27-104]	**0.000**	**0.017**
K02412	fliI; flagellum-specific ATP synthase	425.5 [a]	[265-1148]	468 [a,b]	[251-781]	127.5 [b,c]	[37-258]	79 [c]	[32-112]	**0.001**	**0.017**
K02413	fliJ; flagellar FliJ protein	172.5	[18-447]	408 [a]	[248-789]	129 [b]	[31-164]	70.5 [b]	[29-111]	**0.001**	**0.017**
K02414	fliK; flagellar hook-length control protein FliK	128	[13-438]	354 [a]	[199-602]	65.5 [b]	[9-128]	30.5 [b]	[7-61]	**0.002**	**0.018**
K02416	fliM; flagellar motor switch protein FliM	430.5 [a]	[266-1149]	477 [a,b]	[256-785]	143 [b,c]	[40-266]	87 [c]	[41-125]	**0.001**	**0.017**
K02417	fliNY, fliN; flagellar motor switch protein FliN/FliY	440 [a]	[342-2277]	533	[268-1029]	247.5	[70-425]	180 [b]	[60-262]	**0.006**	**0.021**
K02418	fliOZ, fliO; flagellar protein FliO/FliZ	148	[14-445]	416 [a]	[202-614]	69.5 [b]	[26-131]	48 [b]	[21-69]	**0.001**	**0.017**
K02419	fliP; flagellar biosynthetic protein FliP	436 [a]	[266-1149]	477 [a,b]	[256-793]	143 [b,c]	[40-266]	87.5 [c]	[41-125]	**0.001**	**0.017**
K02420	fliQ; flagellar biosynthetic protein FliQ	431 [a]	[266-1149]	470 [a,b]	[255-789]	141 [b,c]	[40-266]	84.5 [c]	[41-125]	**0.001**	**0.017**
K02421	fliR; flagellar biosynthetic protein FliR	423 [a]	[187-1146]	309.5	[7-460]	69.5 [b]	[23-155]	37.5 [b]	[24-69]	**0.002**	**0.018**
K02422	fliS; flagellar protein FliS	452.5	[291-1147]	551.5	[272-1087]	347.5	[321-501]	350	[189-448]	**0.036**	0.055
K02423	fliT; flagellar protein FliT	108	[10-436]	305 [a]	[2-453]	2	[1-115]	1.5 [b]	[0-33]	**0.005**	**0.020**
K02556	motA; chemotaxis protein MotA	460 [a]	[295-1220]	535.5 [a]	[268-1037]	224	[74-417]	172.5 [b]	[57-251]	**0.001**	**0.017**
K02557	motB; chemotaxis protein MotB	511.5 [a]	[370-1318]	607.5 [a]	[318-1058]	247.5	[157-520]	249 [b]	[84-312]	**0.001**	**0.017**
K13820	fliR-flhB; flagellar biosynthetic protein FliR/FlhB	4.5 [a]	[0-17]	131 [b]	[1-402]	5	[3-76]	7.5	[3-42]	**0.018**	**0.033**

2.3.2 Metabolomics

A total of 533 unique metabolites were identified in the samples. Of these, 334 exhibited spectra that did not match known entries and were subsequently excluded from analysis, while 199 were matched to known compounds.

2.3.2.1 Univariate analysis

After correcting for multiple comparisons, 141 known metabolites were significantly different (q<0.05). The median peak heights and ranges for named metabolites are shown in Table 5.

2.3.2.2 Multivariate analysis

With either PCA or PLS-DA methods, the biochemical composition of the duodenum and ileum were clearly distinct from each other and from that of the large intestinal sites; however, the metabolomic composition of colonic and rectal samples overlapped (Figure 8).

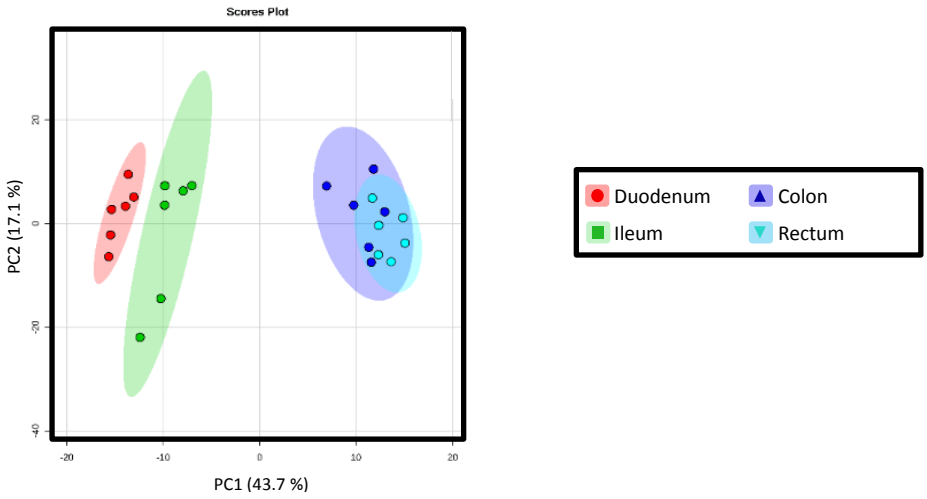


Figure 8. PCA plot showing clustering and variation of the metabolome by location. Shading indicates 95% confidence.

Table 5. Median peak height and range for each named metabolite. *P* and *q* values < 0.05 are shown in bold and are calculated from the transformed and scaled data. Different letters indicate statistically significant differences.

	Duodenum		Ileum		Colon		Rectum			
Compound (BinBase name)	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
1,5-anhydroglucitol	8021.5	[4121-10646]	6949.5	[300-40153]	2478.5	[1132-17553]	3990	[2308-6871]	0.0902	0.1101
1-methylhydantoin	635	[256-1217]	1169.5	[555-6612]	1567.5	[598-3800]	1450.5	[607-4992]	**0.0314**	**0.0446**
1-monoolein	1192	[722-1740]	218.5	[141-5365]	885	[146-2074]	338	[108-974]	0.2717	0.2971
1-monopalmitin	760.5	[421-1069]	643.5	[324-4758]	695	[140-2848]	291.5	[111-1157]	**0.0480**	0.0658
1-monostearin	3321.5	[672-11437]	9300 [a]	[6841-11992]	1044 [b]	[442-2493]	584.5 [b]	[218-3978]	**0.0009**	**0.0030**
2,4-diaminobutyric acid	2514.5 [a]	[1668-3162]	2099 [a]	[1332-6593]	497 [b]	[157-1261]	1226.5	[275-2171]	**0.0020**	**0.0051**
2,5-dihydroxypyrazine NIST	2725.5 [a]	[441-10082]	310 [b]	[162-1844]	294.5 [b]	[67-467]	516.5	[280-780]	**0.0048**	**0.0104**
2,8-dihydroxyquinoline	62 [a]	[25-172]	505 [b]	[290-3359]	219.5	[50-545]	165	[101-212]	**0.0006**	**0.0023**
2'-deoxyguanosine	3051 [a]	[1017-12841]	1905.5	[180-8070]	966.5	[467-2932]	1356 [b]	[163-2528]	**0.0096**	**0.0174**
2-deoxytetronic acid	187 [a]	[103-266]	146.5 [a]	[69-1371]	551	[133-996]	1158 [b]	[415-3626]	**0.0013**	**0.0040**
2-hydroxybutanoic acid	16664.5	[9031-31150]	48357.5 [a]	[8234-86137]	2647.5 [b]	[1327-75320]	4267	[2406-78540]	**0.0088**	**0.0161**
2-hydroxyglutaric acid	208.5 [a]	[61-489]	416	[82-2549]	942.5	[156-3312]	1062.5 [b]	[793-4396]	**0.0056**	**0.0115**
2-hydroxyhexanoic acid	72.5 [a]	[56-195]	880.5 [b]	[319-2790]	662.5	[258-1524]	1786.5 [b]	[272-7599]	**0.0015**	**0.0042**
2-ketobutyric acid	13286.5 [a]	[9973-15702]	13451 [a,b]	[6187-14373]	1035 [c,d]	[668-1358]	1033.5 [b,d]	[735-1266]	**0.0006**	**0.0023**
2-ketoisocaproic acid	2730	[619-11752]	949.5	[707-13700]	707.5	[329-1164]	759.5	[571-1225]	0.0644	0.0817
3-(3-hydroxyphenyl)propionic acid	131 [a]	[50-307]	202.5	[57-1535]	795.5	[78-86228]	16686 [b]	[123-253166]	**0.0049**	**0.0106**
3-(4-hydroxyphenyl)propionic acid	145.5 [a]	[90-264]	171 [a]	[89-1303]	6483	[823-40448]	23939.5 [b]	[3025-76364]	**0.0003**	**0.0023**
3,4-dihydroxycinnamic acid	283 [a]	[139-596]	302.5	[140-637]	548	[114-1982]	1029.5 [b]	[412-1847]	**0.0214**	**0.0328**
3,4-dihydroxyphenylacetic acid	63.5 [a]	[33-73]	71.5	[33-502]	209	[53-816]	705.5 [b]	[51-6343]	**0.0180**	**0.0287**
3,6-anhydro-D-galactose	284.5 [a]	[163-519]	810.5 [b]	[395-5318]	1056	[598-1847]	1175.5 [b]	[777-2363]	**0.0031**	**0.0072**
3-hydroxybutyric acid	2938	[1649-3773]	3377	[1618-20019]	1504	[838-14677]	2723	[931-55693]	0.5519	0.5811
3-phenyllactic acid	53 [a]	[22-140]	549	[217-1264]	791 [b]	[289-5010]	1753.5 [b]	[551-15517]	**0.0011**	**0.0035**
4-aminobutyric acid	9901	[2554-14094]	1662.5	[572-26158]	801.5	[207-12707]	1352	[451-21463]	0.0828	0.1030
4-hydroxybenzoate	403 [a]	[377-543]	834	[578-3031]	919.5	[428-4882]	2797.5 [b]	[830-13501]	**0.0026**	**0.0065**
4-hydroxybutyric acid	222	[130-388]	203.5	[97-2549]	325	[121-1893]	819	[428-15397]	0.0600	0.0780
4-hydroxyphenylacetic acid	46.5 [a]	[23-86]	669.5 [a,b]	[133-1699]	5591.5 [b,c]	[1295-18626]	18122 [c]	[11680-25549]	**0.0001**	**0.0023**
5-aminovaleric acid	13059 [a]	[2926-20179]	80418.5	[10233-498045]	733588.5 [b]	[77538-1257644]	764377.5 [b]	[259335-1586633]	**0.0004**	**0.0023**
5'-deoxy-5'-methylthioadenosine	177	[114-385]	211.5	[30-936]	156	[25-436]	196.5	[73-537]	0.7674	0.7792
5-methoxytryptamine	817.5	[268-2879]	2518 [a]	[1454-3766]	734.5 [b]	[334-1542]	901	[355-2318]	**0.0105**	**0.0180**
6-deoxyglucose	996 [a]	[529-18084]	1497 [a]	[513-9179]	7981.5	[1422-31447]	11444.5 [b]	[6274-31511]	**0.0071**	**0.0138**
7-methylguanine NIST	285.5 [a]	[183-559]	355	[220-1921]	1290 [b]	[511-2916]	1463.5 [b]	[436-2973]	**0.0017**	**0.0046**
adenine	1603.5	[869-3075]	1927.5	[563-9557]	2881	[433-8056]	3863.5	[1355-17040]	0.2607	0.2867

Table 5. (Continued.)

Compound (BinBase name)	Duodenum		Ileum		Colon		Rectum			
	Median	Range	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
adipic acid	367.5	[130-763]	351	[232-3484]	610.5	[188-807]	886	[568-1859]	0.1264	0.1489
alanine	4111125 [a]	[3418094-4971035]	4005396 [a,b]	[3199208-4698134]	298524 [c]	[221832-462232]	364662 [b,c]	[165935-602591]	**0.0006**	**0.0023**
alanine-alanine	36066.5 [a]	[21101-42974]	11721.5	[7332-27451]	16325	[6854-25667]	15866 [b]	[3950-23329]	**0.0189**	**0.0296**
allantoic acid	1369.5	[376-1752]	2358 [a]	[694-21843]	958	[237-1891]	826.5 [b]	[290-1078]	**0.0165**	**0.0264**
alpha-ketoglutarate	60.5 [a]	[40-316]	479.5 [b]	[149-13421]	158.5	[122-307]	197.5	[94-246]	**0.0017**	**0.0045**
aminomalonate	28591.5 [a]	[16837-42089]	9876.5	[3553-35962]	673 [b]	[248-1244]	709.5 [b]	[93-1649]	**0.0003**	**0.0023**
arachidic acid	2092.5	[1796-5200]	1833	[801-17017]	1200.5	[552-4113]	1102	[222-3851]	**0.0103**	**0.0178**
arachidonic acid	3896 [a]	[1433-10136]	2835.5 [a,b]	[490-107237]	343.5 [a,c]	[148-2404]	210 [c]	[108-2772]	**0.0005**	**0.0023**
asparagine	172340.5 [a]	[151905-319135]	46847	[27761-207050]	7907.5 [b]	[3009-11993]	10255.5 [b]	[8045-12762]	**0.0002**	**0.0023**
aspartic acid	1266340.5 [a]	[1037877-1834455]	611972.5 [a,b]	[169383-1794428]	26537 [b,c]	[13263-64661]	13254.5 [c]	[3749-42421]	**0.0001**	**0.0023**
azelaic acid	107 [a]	[70-316]	134.5	[91-994]	225	[48-443]	503.5 [b]	[210-1053]	**0.0078**	**0.0147**
behenic acid	650.5	[457-1125]	947	[285-7345]	554.5	[389-1907]	1208	[643-2377]	0.2053	0.2295
benzoic acid	4596.5	[2929-12174]	3168.5	[2401-41196]	6072	[2245-9645]	4996.5	[4057-10614]	0.8857	0.8857
beta-alanine	1929.5	[713-2753]	729.5	[476-7394]	1223	[292-2655]	2290.5	[790-12012]	0.1665	0.1904
beta-glutamic acid	149.5 [a]	[83-207]	1665.5 [b]	[123-38233]	164	[50-2445]	243	[68-430]	**0.0324**	**0.0457**
beta-glycerolphosphate	1074 [a]	[501-2770]	222	[125-4064]	272.5 [b]	[67-321]	267	[194-348]	**0.0202**	**0.0311**
beta-sitosterol	47.5 [a]	[25-109]	259	[87-637]	362 [b]	[33-4655]	543	[5-796]	**0.0243**	**0.0364**
biphenyl	694	[249-852]	284.5	[54-1254]	288	[157-1665]	1115	[371-1675]	0.0871	0.1070
butyrolactam NIST	1430	[893-1782]	625.5	[448-6729]	936	[213-4321]	1656	[590-4508]	0.6685	0.6929
capric acid	408.5	[266-680]	354	[270-5270]	389.5	[167-1220]	420	[356-1217]	0.5821	0.6097
caprylic acid	4499.5 [a]	[1309-9324]	1678.5	[1162-17249]	1342 [b]	[410-3717]	1540.5	[1005-3957]	**0.0228**	**0.0346**
cellobiose	215.5	[87-356]	212	[65-434]	89	[28-392]	211.5	[35-1018]	0.2007	0.2256
cholesterol	1975.5	[720-4294]	1651 [a]	[1179-27408]	805.5	[406-3273]	531.5 [b]	[286-2869]	**0.0037**	**0.0082**
citric acid	272	[104-1479]	2079 [a]	[903-11853]	148 [b]	[49-607]	106 [b]	[45-419]	**0.0003**	**0.0023**
citrulline	18504.5 [a,b]	[14239-24836]	18871 [b]	[18127-23464]	1989 [c]	[1313-9651]	2803 [a,c]	[2128-7512]	**0.0004**	**0.0023**
creatinine	103298.5 [a]	[49704-195215]	23067.5 [a,b]	[8256-205774]	2141.5 [c]	[868-12850]	5036 [b,c]	[1035-21389]	**0.0006**	**0.0023**
cystathionine NIST	2240.5	[411-3108]	1856.5	[981-8295]	1624	[516-5106]	6569	[1298-15718]	0.0845	0.1045
cysteine	224942.5 [a]	[50059-459113]	41293.5	[12193-75133]	4065.5 [b]	[2723-9372]	4207 [b]	[2192-9777]	**0.0002**	**0.0023**
cysteine-glycine	2544 [a]	[405-7118]	648	[232-1959]	468 [b]	[234-1158]	567	[382-715]	**0.0252**	**0.0372**
cystine	161825 [a]	[88272-316694]	55098	[25563-165395]	370.5 [b]	[241-730]	377.5 [b]	[235-681]	**0.0003**	**0.0023**
cytidine	19997.5 [a]	[9190-30522]	1319.5	[351-6689]	219.5 [b]	[101-864]	202 [b]	[75-695]	**0.0002**	**0.0023**
cytosine	12466 [a]	[7952-33374]	2565	[1282-6934]	695.5 [b]	[352-1469]	606 [b]	[410-1163]	**0.0002**	**0.0023**
dehydroascorbic acid	28296.5 [a]	[8389-45183]	8530.5	[387-24695]	2263.5 [b]	[201-8794]	1867 [b]	[485-11034]	**0.0074**	**0.0142**

Table 5. (Continued.)

Compound (BinBase name)	Duodenum		Ileum		Colon		Rectum			
	Median	Range	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
D-erythro-sphingosine	459.5	[167-571]	737.5 [a]	[312-2684]	192 [b]	[94-1583]	213 [b]	[137-339]	**0.0081**	**0.0152**
digalacturonic acid	75.5	[40-153]	369	[56-1911]	60.5	[19-1254]	136	[60-177]	0.0546	0.0719
dihydroxyacetone	2507.5 [a]	[945-7797]	561	[504-7606]	841 [b]	[320-1851]	1145	[930-1902]	**0.0230**	**0.0347**
erythritol	913.5	[419-1606]	557	[417-5869]	1213.5	[179-4481]	1571	[312-11471]	0.5074	0.5371
ethanolamine	1992733 [a]	[1148597-4029936]	647912	[352641-3309640]	21534 [b]	[6351-46816]	18174 [b]	[10122-32607]	**0.0004**	**0.0023**
ferulic acid	816.5	[40-1174]	318.5	[87-772]	799.5	[180-1251]	928	[169-1812]	0.1020	0.1222
fructose	1356 [a]	[773-2338]	169 [b]	[54-3475]	413	[147-1514]	735.5	[382-947]	**0.0099**	**0.0176**
fucose	6346 [a]	[3536-21041]	8979	[933-104119]	48706.5 [b]	[17241-234088]	25924.5	[7635-43896]	**0.0103**	**0.0178**
fumaric acid	15645.5 [a]	[8822-39110]	10845.5	[1590-96261]	1767.5 [b]	[719-2953]	1604 [b]	[1329-2177]	**0.0010**	**0.0033**
galactinol	239 [a]	[88-868]	1452 [b]	[732-5552]	517.5	[79-3376]	341.5 [a]	[88-526]	**0.0115**	**0.0192**
galactose	7895	[2911-28987]	6252	[1243-128539]	31672.5	[9900-42752]	15321.5	[3037-24432]	0.1485	0.1718
galacturonic acid	2414.5 [a]	[1040-5081]	1334.5	[802-3040]	685.5	[67-6687]	302.5 [b]	[163-352]	**0.0056**	**0.0115**
glucoheptulose	208 [a]	[78-452]	1534.5 [b]	[322-3892]	249	[104-583]	174.5 [a]	[63-599]	**0.0023**	**0.0060**
gluconic acid	304.5 [a]	[189-778]	121.5	[48-820]	52 [b]	[33-68]	92 [b]	[44-120]	**0.0009**	**0.0030**
glucose	5984.5	[1692-18446]	5642	[3488-26495]	9980.5	[6677-62441]	24508.5	[4547-40026]	**0.0195**	**0.0303**
glucose-1-phosphate	1786.5	[491-2991]	5004 [a]	[2027-10521]	1305.5 [b]	[259-1772]	880.5 [b]	[506-2192]	**0.0028**	**0.0068**
glutamic acid	1709486 [a]	[1026840-2226949]	1794420 [a,b]	[1514715-2259318]	117028 [a,c]	[59336-491284]	99331 [c]	[48763-441273]	**0.0005**	**0.0023**
glutamine	211543 [a]	[150014-978152]	171473 [a,b]	[106889-455724]	3992.5 [c]	[1658-50339]	6121.5 [b,c]	[2561-41768]	**0.0006**	**0.0023**
glutamyl-valine	2019 [a]	[752-3049]	1913.5 [a]	[185-8761]	304	[97-1617]	223 [b]	[82-310]	**0.0010**	**0.0033**
glutaric acid	91.5 [a]	[23-234]	286	[95-1161]	386	[151-612]	902 [b]	[123-1476]	**0.0094**	**0.0171**
glyceric acid	2821.5	[1220-7745]	3758	[860-21525]	3387	[1255-8064]	2388	[1572-10006]	0.8511	0.8597
glycerol	790358 [a]	[335204-1175073]	266781.5	[258859-3323888]	17329 [b]	[9333-38553]	15393 [b]	[10718-37086]	**0.0005**	**0.0023**
glycerol-3-galactoside	2328.5 [a,b]	[1046-4574]	3703 [b]	[1866-11110]	699.5 [c]	[264-935]	807 [a,c]	[418-958]	**0.0005**	**0.0023**
glycerol-alpha-phosphate	21013 [a]	[10192-34413]	3392.5	[987-57006]	2121 [b]	[716-2966]	831.5 [b]	[461-2073]	**0.0009**	**0.0030**
glycine	930615 [a]	[640214-1895054]	934944.5 [a,b]	[661892-1777776]	19043.5 [c]	[10515-33287]	24799.5 [b,c]	[8896-58494]	**0.0005**	**0.0023**
glycolic acid	1720 [a]	[1138-3887]	1811	[988-18909]	10519.5	[3242-14855]	8381.5 [b]	[4160-16002]	**0.0061**	**0.0121**
glycyl tyrosine	2001 [a]	[933-3209]	2777.5 [a,b]	[801-5316]	515.5 [a,c]	[380-1299]	321 [c]	[126-949]	**0.0007**	**0.0026**
gly-pro	261 [a]	[89-433]	694 [b]	[154-1352]	343	[139-1822]	330.5	[238-1141]	**0.0037**	**0.0082**
guanine	229 [a]	[133-297]	1590 [b]	[647-2857]	861 [b]	[545-1433]	606.5	[403-873]	**0.0004**	**0.0023**
guanosine	2159.5 [a]	[1382-15528]	2530	[1199-5110]	2067.5	[180-4696]	1564.5 [b]	[86-4273]	**0.0185**	**0.0292**
heptadecanoic acid	1512 [a]	[997-4208]	4795.5 [b]	[3813-50192]	2478	[806-6515]	1598.5	[1217-7012]	**0.0028**	**0.0068**
hexitol	3458	[1466-12244]	14338.5 [a]	[6714-56112]	328 [b]	[177-1717]	752 [b]	[182-1139]	**0.0003**	**0.0023**
hexonic acid	563.5	[344-1352]	509.5	[158-1197]	441	[169-707]	539.5	[248-861]	0.3403	0.3680

Table 5. (Continued.)

Compound (BinBase name)	Duodenum		Ileum		Colon		Rectum			
	Median	Range	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
hexuronic acid	28037 [a]	[3163-48933]	38113.5 [a]	[14434-88434]	2835	[356-28849]	386 [b]	[342-2931]	**0.0006**	**0.0023**
histidine	325157.5 [a,b]	[162639-433843]	511451.5 [b]	[148610-898975]	14196 [c]	[9321-25535]	15104.5 [a,c]	[11058-23506]	**0.0005**	**0.0023**
homocystine	477.5	[249-625]	175.5	[86-1023]	177.5	[66-1649]	547.5	[139-1746]	0.1515	0.1743
homoserine	248.5 [a]	[109-349]	634	[249-2120]	1187 [b]	[394-8132]	1372 [b]	[633-7995]	**0.0014**	**0.0042**
hydroxycarbamate NIST	4721.5	[3340-10473]	4242	[2014-76378]	5036.5	[3169-12575]	5271	[4526-13691]	0.7658	0.7792
hydroxylamine	35056.5	[24996-65994]	70192.5	[25134-482450]	35430	[24334-77172]	35730.5	[23545-61885]	0.0633	0.0813
hypoxanthine	21793.5	[3178-67353]	35704	[6126-80172]	11441.5	[4868-33432]	5848	[1073-11674]	**0.0416**	0.0578
indole-3-acetate	91 [a]	[33-163]	190 [a,b]	[65-936]	2261 [b,c]	[278-5488]	5501.5 [c]	[1895-11132]	**0.0002**	**0.0023**
inosine	5234.5	[2365-17450]	1367.5	[802-2622]	4226.5	[154-22026]	2546	[28-12745]	0.0644	0.0817
inositol-4-monophosphate	143.5	[81-1169]	60.5	[21-2847]	109	[11-369]	115	[57-283]	0.3122	0.3395
isoleucine	1111779.5 [a]	[801644-1841393]	2005640.5 [a,b]	[651199-2179639]	88500.5 [a,c]	[41500-162526]	66291.5 [c]	[23537-127145]	**0.0004**	**0.0023**
isothreonic acid	408.5	[232-621]	259.5	[181-3137]	425.5	[216-580]	377	[297-1230]	0.8763	0.8808
lactamide	501 [a]	[227-1146]	250	[205-2436]	94	[24-644]	60 [b]	[34-645]	**0.0052**	**0.0110**
lactose	747 [a]	[400-1163]	291.5	[110-1226]	145.5	[54-1443]	169 [b]	[58-260]	**0.0065**	**0.0128**
leucine	2601243 [a]	[889141-3265881]	3151465.5 [a,b]	[1956352-4195069]	145389.5 [a,c]	[72205-500255]	122537 [c]	[48012-265958]	**0.0003**	**0.0023**
levoglucosan	516	[289-666]	1420 [a]	[599-2761]	257 [b]	[59-873]	479	[139-1578]	**0.0033**	**0.0075**
linoleic acid	1780.5 [a]	[506-4231]	935.5 [a]	[190-31061]	155.5 [b]	[69-1160]	648	[104-1141]	**0.0059**	**0.0118**
lysine	1130780 [a]	[614935-1454012]	531333 [a,b]	[214400-1571569]	49501 [c]	[40933-178443]	108815.5 [b,c]	[67049-153195]	**0.0003**	**0.0023**
lyxitol	5135	[325-44013]	2359	[1996-11742]	1523	[232-5770]	1922	[1256-9714]	0.6063	0.6317
lyxose	3026	[515-4465]	4297.5	[530-5226]	1233	[506-4353]	2600.5	[1127-6938]	0.3449	0.3710
maleimide	16299 [a]	[10712-22560]	6799	[1143-36119]	2681.5 [b]	[1362-3495]	2340 [b]	[1887-3740]	**0.0055**	**0.0114**
malic acid	30274 [a]	[17599-69988]	8168 [a,b]	[1646-145082]	2492 [b,c]	[990-3622]	1082 [c]	[371-2232]	**0.0004**	**0.0023**
maltose	659.5	[386-10378]	3123.5	[773-15521]	3058	[1378-4720]	4947	[1738-7158]	0.0620	0.0801
maltotriose	31.5 [a]	[17-98]	50.5	[23-347]	67.5	[40-160]	137.5 [b]	[45-385]	**0.0106**	**0.0180**
mannose	3663	[2028-6079]	2681.5	[629-15926]	2299	[1131-8109]	1701.5	[562-3612]	0.1996	0.2256
methanolphosphate	4529	[1918-77223]	1111.5	[337-3824]	623	[198-8060]	3142	[155-3944]	0.0525	0.0696
methionine	452897.5 [a]	[287428-824338]	402933.5 [a,b]	[179735-452617]	14872.5 [b,c]	[8732-27359]	13138 [c]	[6987-21476]	**0.0004**	**0.0023**
methionine sulfoxide	8268.5	[4834-15240]	11720 [a]	[6700-32470]	4177 [b]	[2312-10378]	4981.5 [b]	[2637-7333]	**0.0085**	**0.0158**
methyl O-D-galactopyranoside	2413.5 [a]	[1421-3351]	4468	[1682-14430]	8643 [b]	[2720-73587]	13001 [b]	[3420-22998]	**0.0024**	**0.0060**
methyltetrahydrophenanthrenone NIST	556	[282-1146]	530	[364-5087]	607.5	[220-1292]	959.5	[449-1230]	0.4494	0.4782
myristic acid	5179	[3107-6547]	3392.5	[1438-63637]	8228	[2097-20141]	17074.5	[7604-24637]	0.0951	0.1147
N-acetylaspartic acid	625	[428-774]	1411.5	[797-2403]	1328.5	[503-2450]	1506	[235-2376]	**0.0396**	0.0555
N-acetyl-D-galactosamine	3704.5	[1583-10732]	3117 [a]	[67-4188]	6296 [b]	[3317-29693]	5145	[103-12807]	**0.0263**	**0.0385**

Table 5. (Continued.)

Compound (BinBase name)	Duodenum		Ileum		Colon		Rectum			
	Median	Range	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
N-acetyl-D-hexosamine	2957 [a]	[796-5610]	914	[412-6239]	520 [b]	[435-2169]	793.5 [b]	[190-1000]	**0.0073**	**0.0140**
N-acetyl-L-ornithine	278.5	[104-711]	546.5	[115-29597]	1103	[411-4253]	1631.5	[598-2034]	**0.0496**	0.0667
N-acetylputrescine	16428.5	[839-24791]	10865	[3357-21998]	2259	[521-11561]	2812.5	[1847-8834]	**0.0305**	**0.0436**
n-epsilon-trimethyllysine	1141 [a]	[803-2166]	977	[267-1613]	295.5 [b]	[98-917]	298 [b]	[206-723]	**0.0031**	**0.0072**
nicotinamide	10850 [a]	[4185-24701]	627.5	[83-15106]	406 [b]	[233-1568]	1402	[347-2355]	**0.0035**	**0.0078**
nicotinic acid	629.5 [a]	[168-1755]	8385.5 [b]	[5003-25499]	11928.5 [b]	[3061-33017]	14785.5 [b]	[3120-25906]	**0.0046**	**0.0100**
N-methylalanine	8577.5 [a]	[4439-20288]	42154	[13040-82229]	51530	[7622-230309]	61799 [b]	[11724-193811]	**0.0151**	**0.0247**
norvaline	871	[263-2028]	1845	[428-3294]	3374	[1108-44420]	2817	[1110-36677]	**0.0496**	0.0667
ornithine	312532 [a]	[77883-491450]	264649.5 [a]	[110411-555802]	9798 [b]	[7208-50796]	19651	[7067-29179]	**0.0005**	**0.0023**
orotic acid	274	[129-550]	448 [a]	[99-7143]	65 [b]	[35-186]	82.5 [b]	[24-166]	**0.0011**	**0.0035**
oxalic acid	203	[91-1506]	970.5	[485-4932]	112.5	[45-3522]	102	[39-3713]	0.0600	0.0780
oxoproline	924410.5 [a]	[631837-1174316]	882320.5 [a]	[545482-1082347]	36417 [b]	[30843-144935]	35600.5 [b]	[22273-187653]	**0.0006**	**0.0023**
palmitic acid	17185.5	[10656-33298]	22031.5	[13600-282551]	17634	[7578-39361]	21358	[14890-48100]	0.2565	0.2835
pantothenic acid	2356.5	[752-3508]	1408.5	[528-4672]	1841	[986-4359]	2619.5	[738-3902]	0.7642	0.7792
pelargonic acid	6156.5	[2577-10304]	8556.5	[6294-105451]	9157.5	[2979-25705]	13467	[5830-28692]	0.0511	0.0682
pentadecanoic acid	1543	[186-4309]	2929.5	[1177-28088]	2265.5	[821-5841]	2691.5	[1718-4339]	0.1075	0.1281
phenylacetic acid	538.5 [a]	[444-945]	561 [a]	[426-975]	2790	[107-11906]	3227.5 [b]	[1810-13417]	**0.0140**	**0.0230**
phenylalanine	1066226.5 [a]	[574792-1621399]	1450835 [a,b]	[544207-1852113]	114471.5 [a,c]	[29019-249126]	100982 [c]	[30988-187156]	**0.0005**	**0.0023**
phenylethylamine	915	[470-2468]	380 [a]	[63-1255]	4050.5 [b]	[798-25688]	3479.5 [b]	[729-26899]	**0.0005**	**0.0023**
phenylpyruvate	249 [a]	[148-766]	460	[292-830]	423	[226-2742]	987 [b]	[491-8261]	**0.0130**	**0.0215**
phosphate	413261 [a]	[276526-1009730]	205953 [a,b]	[74456-1001565]	1926 [b,c]	[847-8363]	1357.5 [c]	[1104-1947]	**0.0003**	**0.0023**
phosphoethanolamine	637 [a]	[236-958]	119.5	[74-569]	61 [b]	[36-152]	97 [b]	[40-126]	**0.0012**	**0.0037**
pinitol	77.5	[40-1185]	122	[62-3264]	65	[11-182]	127	[51-1421]	0.1468	0.1709
pipecolinic acid	92.5 [a]	[13-335]	194.5	[90-1351]	562	[205-4031]	4171.5 [b]	[1190-11932]	**0.0015**	**0.0042**
piperidone	633 [a]	[319-1017]	953.5 [a,b]	[411-3842]	4619 [b,c]	[1032-9167]	11369.5 [c]	[2108-22865]	**0.0002**	**0.0023**
proline	1938180.5 [a]	[1239458-3016264]	2445473 [a]	[1416201-2837691]	34900 [b]	[25082-94877]	34712 [b]	[16540-99534]	**0.0006**	**0.0023**
propane-1,3-diol NIST	1851	[742-5298]	3234.5	[716-12791]	1480.5	[567-3374]	1766	[1000-4830]	0.7270	0.7496
pseudo uridine	9486 [a]	[4267-13302]	5831.5	[3586-11568]	4015	[1090-7556]	3293 [b]	[896-5488]	**0.0109**	**0.0184**
putrescine	1297 [a]	[602-2774]	30871.5	[2191-167327]	267989 [b]	[130599-935794]	341061.5 [b]	[107769-1161861]	**0.0002**	**0.0023**
pyruvic acid	1051.5 [a,b]	[593-2807]	9536.5 [c]	[5745-16720]	2228.5 [b,d]	[884-3342]	4824 [c,d]	[2811-5646]	**0.0002**	**0.0023**
quinic acid	73 [a]	[21-105]	146	[39-985]	233.5	[57-719]	849 [b]	[416-1190]	**0.0015**	**0.0042**
ribitol	22574.5 [a]	[6715-45932]	2386	[1894-23764]	1572 [b]	[662-10142]	1422 [b]	[288-2606]	**0.0026**	**0.0065**
ribonic acid	1499	[551-3154]	722.5	[403-3909]	553	[132-1095]	530.5	[328-1539]	0.0676	0.0846
ribose	36292	[7426-63140]	5462.5	[3585-52035]	14972.5	[4705-70419]	15627	[2888-47200]	0.1419	0.1661

Table 5. (Continued.)

Compound (BinBase name)	Duodenum		Ileum		Colon		Rectum			
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
serine	1417440 [a]	[1081314-1900474]	444285	[164381-923030]	21513 [b]	[12005-38304]	16968 [b]	[10785-32899]	**0.0002**	**0.0023**
sinapinic acid	46.5 [a]	[30-122]	68	[20-946]	347.5	[60-612]	359.5 [b]	[258-856]	**0.0053**	**0.0111**
sorbitol	3985.5	[2378-12062]	5678.5	[2617-8256]	1714.5	[467-6584]	3314.5	[732-8973]	0.1087	0.1288
spermidine	1571 [a]	[862-1854]	3993.5	[2664-12346]	3193.5	[1271-22057]	15487.5 [b]	[3882-41319]	**0.0017**	**0.0045**
spermine	2413 [a]	[1153-8803]	2001	[1302-5025]	683.5 [b]	[160-2765]	1027.5	[385-2453]	**0.0153**	**0.0247**
stearic acid	83265.5	[49726-178526]	132847	[73800-1647885]	81754.5	[43924-199756]	101003.5	[68694-235232]	**0.0292**	**0.0421**
succinate semialdehyde	1182.5 [a]	[544-2503]	799.5 [a,b]	[284-1315]	151 [c]	[70-281]	222 [b,c]	[117-336]	**0.0004**	**0.0023**
succinic acid	21900	[17604-34484]	411074	[10564-896930]	16616.5	[831-875515]	4845.5	[1245-196187]	0.0664	0.0836
sulfuric acid	491.5	[101-8309]	5451 [a]	[481-9004]	390.5	[35-3231]	68 [b]	[24-179]	**0.0007**	**0.0025**
taurine	80674.5	[40417-132110]	329901 [a]	[54489-919201]	72561	[476-129767]	5250.5 [b]	[322-62095]	**0.0030**	**0.0071**
threitol	747	[240-2529]	223.5	[134-3224]	246.5	[85-905]	398.5	[159-1050]	0.3476	0.3719
threonic acid	479 [a,b]	[344-1859]	749.5 [b]	[329-9807]	190 [c]	[90-486]	283 [a,c]	[122-454]	**0.0012**	**0.0038**
threonine	808060 [a]	[528852-877182]	421047.5 [a,b]	[296471-987264]	22659 [b,c]	[10481-44287]	18257.5 [c]	[7458-49781]	**0.0003**	**0.0023**
thymidine	16430 [a]	[9935-34923]	10133 [a,b]	[2195-26498]	3083.5 [b,c]	[610-4266]	1269 [c]	[322-3189]	**0.0004**	**0.0023**
thymine	21868.5 [a]	[12468-51948]	29266.5 [a]	[10831-49208]	10000.5	[2730-21895]	9004 [b]	[3343-11361]	**0.0027**	**0.0066**
trans-4-hydroxyproline	270.5	[117-433]	168.5	[102-2355]	740.5	[60-949]	2167	[409-6277]	**0.0427**	0.0590
tryptophan	471778.5 [a,b]	[297445-923000]	686598 [b]	[329824-1005368]	71302.5 [c]	[23990-135134]	110766 [a,c]	[24544-161584]	**0.0005**	**0.0023**
tyrosine	1791955.5 [a]	[1104069-2345118]	2090782 [a]	[803070-2293451]	168617.5 [b]	[74737-293816]	146435 [b]	[57832-312115]	**0.0006**	**0.0023**
UDP GlcNAc	57	[14-109]	138.5	[42-521]	54	[26-67]	86	[62-106]	**0.0249**	**0.0369**
UDP-glucuronic acid	2873.5	[186-11309]	6474 [a]	[1682-25163]	1512	[293-8136]	539.5 [b]	[218-4041]	**0.0100**	**0.0177**
uracil	28583	[18314-87961]	49102	[30983-173299]	23478.5	[7627-58048]	29785	[8229-126885]	0.0951	0.1147
urea	113154 [a]	[65856-213498]	31410	[12777-91404]	2173.5 [b]	[32-3041]	2136.5 [b]	[1685-5171]	**0.0002**	**0.0023**
uric acid	154943.5 [a]	[122073-191054]	53608	[7743-641297]	911.5 [b]	[166-2478]	1007 [b]	[222-2198]	**0.0005**	**0.0023**
uridine	3155.5 [a]	[2068-14643]	3014.5	[763-6515]	3481	[435-5984]	834.5 [b]	[352-4457]	**0.0272**	**0.0394**
urocanic acid	1436	[249-2522]	667	[460-1766]	773.5	[99-1358]	458.5	[152-914]	0.1757	0.1998
valine	2424533 [a]	[1689230-2930533]	2351440.5 [a,b]	[1645335-3448589]	145794.5 [a,c]	[77598-252763]	135537 [c]	[40757-243482]	**0.0005**	**0.0023**
vanillic acid	92 [a]	[28-226]	155.5	[65-1120]	255	[72-1028]	555.5 [b]	[386-2474]	**0.0014**	**0.0042**
xanthine	89616 [a]	[15556-159664]	43978 [a,b]	[31998-50746]	8642 [b,c]	[2162-21217]	7788.5 [c]	[2247-14368]	**0.0007**	**0.0026**
xanthosine	1954.5 [a]	[288-4128]	704.5	[370-1708]	188.5 [b]	[64-346]	180.5 [b]	[37-482]	**0.0015**	**0.0042**
xanthurenic acid	110.5 [a]	[26-627]	654 [b]	[222-1193]	87 [a]	[25-1419]	159	[96-428]	**0.0097**	**0.0174**
xylitol	15052.5 [a]	[7131-39878]	3056.5	[2129-27663]	365 [b]	[61-1395]	308 [b]	[89-1389]	**0.0004**	**0.0023**
xylonolactone NIST	207.5	[94-5516]	1498.5	[876-2255]	1082	[113-3095]	1927.5	[165-3767]	0.2523	0.2804
xylose	3455 [a]	[1508-6118]	6530	[2848-15955]	29602 [b]	[9960-73627]	43137.5 [b]	[8057-102445]	**0.0003**	**0.0023**
xylulose NIST	6299	[4766-8723]	3727	[1021-22548]	2036	[1271-5940]	2274	[764-3955]	**0.0491**	0.0667

PLS-DA, a supervised method, can also be used to identify which metabolites contribute most significantly to the separation of groups. The significance is graphed by VIP scores and the highest scoring metabolites were subsequently used to generate a heatmap to visually highlight the patterns across samples within and between gastrointestinal sites (Figure 9). The top fifteen features identified by VIP scores were cystine, phosphate, 4-hydroxyphenylacetic acid, uric acid, putrescine, ethanolamine, aspartic acid, 3-(4-hydroxyphenyl)propionic acid, serine, proline, aminomalonate, threonine, cytidine, glycine, and 5-aminovaleric acid (Figure 10A).

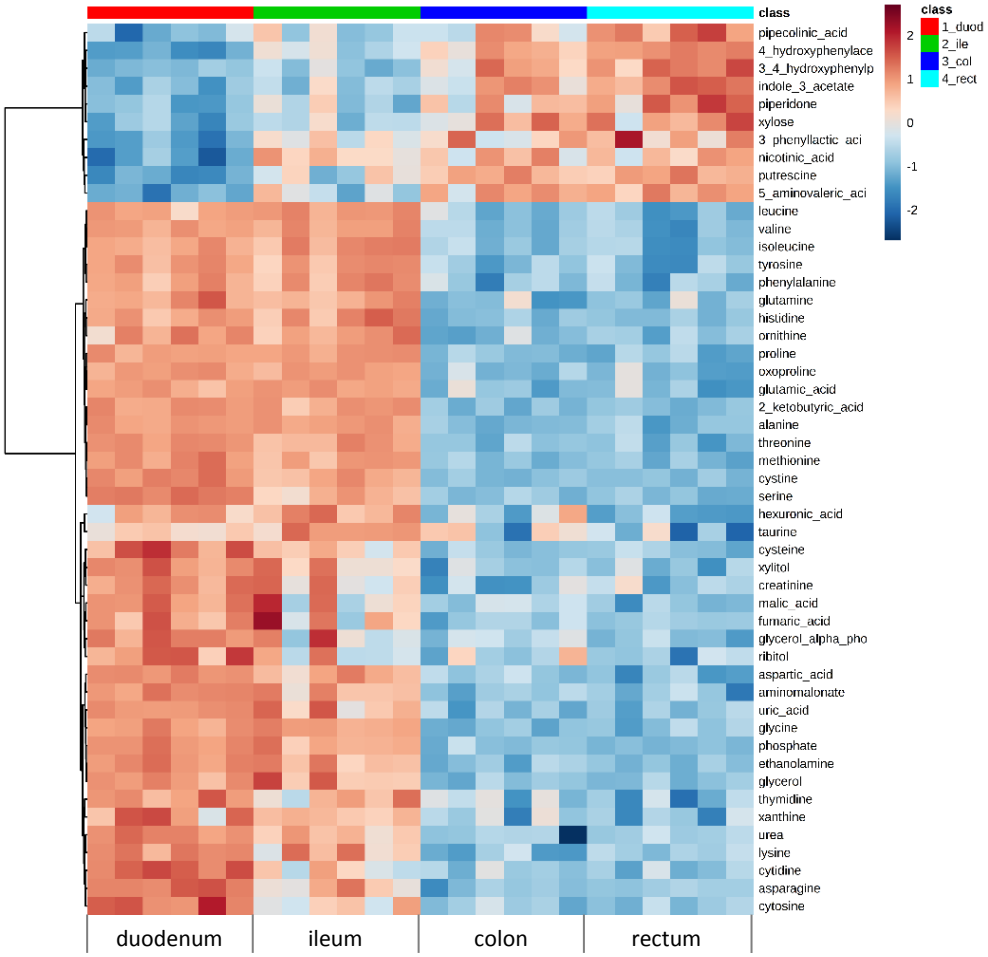


Figure 9. Heatmap showing the relative distribution of the top 50 metabolites, as identified by VIP scores in PLS-DA. Each column represents an individual sample and samples are grouped by organ site along the x-axis.

Random Forest Analysis is another supervised method for finding the most significant features. The importance of each feature is determined by how significantly the classification accuracy is lost when that feature is not used in the model. The top fifteen metabolites identified using this method were cytidine, pyruvic acid, 4-hydroxyphenylacetic acid, cytosine, serine, xylitol, 2-hydroxyhexanoic acid, aminomalonate, aspartic acid, nicotinic acid, phosphate, ethanolamine, 3-phenyllactic acid, guanine, and valine (Figure 10B).

2.3.2.3 Examples of metabolite profiles

Although untargeted metabolomics does not provide quantitative results for any given metabolite, the normalized peak height for any specific metabolite across all of the samples can be considered semi-quantitative. Individual metabolites that did significantly change along the GIT were subjectively found to exhibit one of four patterns: incrementally increasing or decreasing along the length, abruptly decreasing from ileum to colon, and rarely, an increase with a subsequent decrease. Examples of each of these patterns are shown in Figure 11.

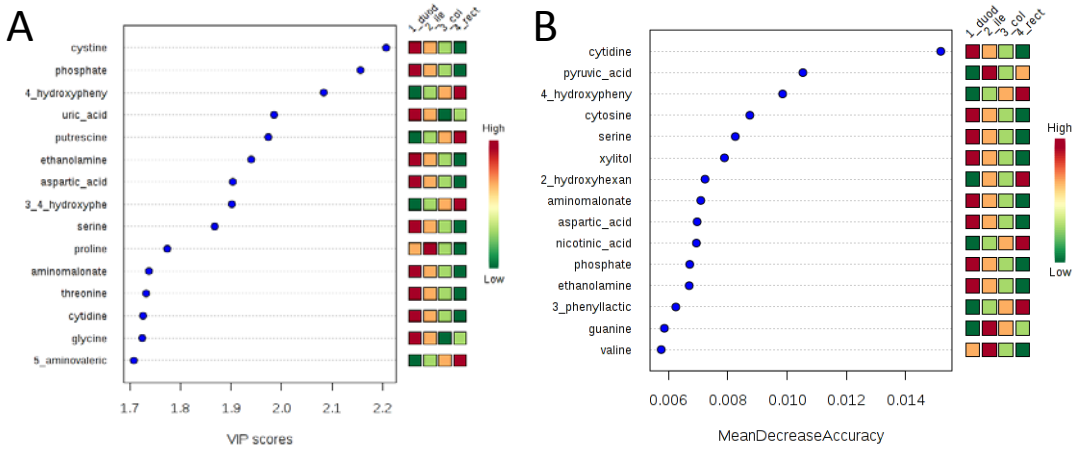


Figure 10. Comparison of the top 15 metabolites as identified by (A) PLS-DA or (B) Random Forest Analysis.

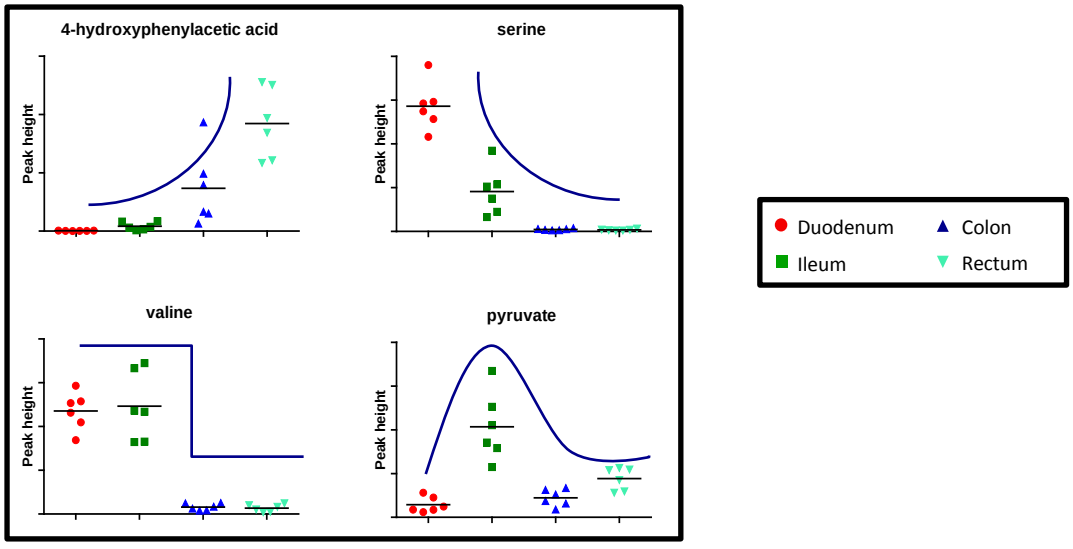


Figure 11. Examples of semi-quantitative metabolite profiles observed in the metabolomics data set. Lines are fit subjectively.

2.3.3 Inter-omics results

Prior to filtering correlation scores, the table consisted of 199 named metabolites and 34 bacterial families. At a stringent threshold of rho ≥0.8, 19 metabolites and four bacterial families were included (Table 6). The bacterial families were Lachnospiraceae, Peptococcaceae, Veillonellaceae, and an unspecified family-level taxon within Clostridiales. The metabolites were 2-ketobutyric acid, 3-(3-hydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl)propionic acid, alanine, cystine, ethanolamine, glutamic acid, glycerol, glycine, hexitol, histidine, isoleucine, leucine, methionine, oxoproline, phosphate, proline, threonine, and valine. With the exception of 3-(3-hydroxyphenyl)propionic acid and 3-(4-hydroxyphenyl)propionic acid, all were negatively correlated with the bacterial families.

Table 6. Pearson correlation matrix between named metabolites and bacterial families with at least one absolute rho ≥ ±0.8.

Metabolite	unspecified Clostridiales	Lachnospiraceae	Peptococcaceae	Veillonellaceae
2-ketobutyric acid	-0.79	-0.85	-0.49	-0.65
3-(3-hydroxyphenyl)propionic acid	0.36	0.45	0.83	0.63
3-(4-hydroxyphenyl)propionic acid	0.66	0.74	0.64	0.80
alanine	-0.79	-0.85	-0.59	-0.69
cystine	-0.76	-0.82	-0.52	-0.63
ethanolamine	-0.74	-0.82	-0.48	-0.55
glutamic acid	-0.80	-0.81	-0.52	-0.75
glycerol	-0.74	-0.81	-0.37	-0.49
glycine	-0.79	-0.84	-0.43	-0.57
hexitol	-0.76	-0.84	-0.54	-0.65
histidine	-0.78	-0.84	-0.45	-0.69
isoleucine	-0.76	-0.81	-0.58	-0.76
leucine	-0.79	-0.82	-0.65	-0.74
methionine	-0.76	-0.80	-0.55	-0.71
oxoproline	-0.78	-0.81	-0.54	-0.72
phosphate	-0.78	-0.83	-0.54	-0.66
proline	-0.85	-0.87	-0.51	-0.73
threonine	-0.75	-0.80	-0.53	-0.69
valine	-0.73	-0.81	-0.58	-0.75

2.4 DISCUSSION

2.4.1 Microbiota composition

Suchodolski et al. (2008) sampled contents of duodenum, jejunum, ileum, and colon and identified four predominant phyla within the healthy canine GIT using 16S rRNA gene clone libraries: Firmicutes, Fusobacteria, Bacteroidetes, and Proteobacteria. Percentages of Proteobacteria were driven primarily by Enterobacteriales, and decreased from 32% in the duodenum to 1.4% in the colon. The order Clostridiales was a major constituent at all four sites, and the overall most abundant order in the duodenum and jejunum. Sequences assigned to Clostridium cluster XI were more abundant in the small intestine, while sequences of Clostridium cluster XIVa were more abundant in the colon. Although the current study sampled duodenum, ileum, colon, and rectum, the results are consistent with the previous findings. Sequences belonging to Actinobacteria were identified in addition to the four previously mentioned phyla, but contributed minimally at each site. Proteobacteria predominated in the duodenum with 59% of sequences, and decreased to 5% in the rectum. The proportionate increase in Lachnospiraceae and Ruminococcaceae in the large intestine versus small intestine is also consistent with Suchodolski et al. (2008), describing increased Clostridium cluster XIVa in the colon. Firmicutes, Proteobacteria, and Bacteroidetes have previously been reported as predominant phyla in the GIT of other mammals, including cattle, sheep, pigs, and humans (Mao et al. 2015; J. Wang et al. 2017; H. Yang et al. 2016; M. Wang et al. 2005; G. Li et al. 2015).

2.4.2 Community metabolic potential

While PICRUSt was originally validated for human-derived data sets, the applicability of this tool for use with canine-derived sequencing data compared to human-derived data has been described by Vazquez-Baeza et al. (2016). PICRUSt analysis identified significant differences in

KEGG pathway categories that appear irrelevant to the microbiota. It is likely that each of these categories was driven to significance by relatively few KOs that are associated with human diseases but also are present in bacteria, hence originating from the 16S rRNA sequencing data. This limitation also manifests in the reduction of KOs that successfully mapped to KEGG pathways, where any given KO of bacterial origin may not be completely annotated to a specific KEGG pathway.

PICRUSt also revealed significant differences in bile acid synthesis pathways. This was expected since the role of microbiota in deconjugating and dehydroxylating bile acids is well established. The abundance of predicted gene copies orthologous to choloylglycine hydrolase significantly increased along the GIT, consistent with deconjugation of bile acids occurring primarily in the large intestine. The second significantly altered KO associated with bile acid synthesis encodes alpha-methylacyl-CoA racemase. The function of this enzyme is described as inversion of a stereocenter during synthesis of primary bile acids from cholesterol; however, as bacteria modify bile acids but do not synthesize them, it may be suggested that this enzyme in bacteria may bind to the same substrate, and perform the same biochemical conversion, but for an alternative purpose than synthesis of primary bile acids.

The flagellar assembly pathway consists of proteins of bacterial origin, yet flagellum is also relevant to the host immune system (Cullender et al. 2013; Haiko and Westerlund-Wikstrom 2013). The data showed increased abundance of KOs associated with the flagellar assembly in the small versus large intestine. Although metabolic capacity of the community is not equivalent to expression, these results highlight the differing ecosystems between sites in the gut.

2.4.3 Metabolomics profiling

The simplest analysis of metabolites one-by-one is represented in Table 5. As with any large data set, different statistical methods have different biases. Therefore, multivariate analysis

encompassed both PLS-DA and RFA to identify metabolites that consistently demonstrated significance. Also, findings in this study may partially depend on diet (as some metabolites may only be present in the GIT if certain ingredients are incorporated in the diet). An exhaustive discussion of the overlap of all significant metabolites by both methods is likely not warranted given the limitations of this study, so only the top fifteen metabolites identified by each method (an arbitrary cutoff) were considered for further discussion. This resulted in seven metabolites appearing in both lists: phosphate, 4-hydroxyphenylacetic acid, ethanolamine, aspartic acid, serine, aminomalonate, and cytidine. The GIT is credited with a critical role in phosphate homeostasis in partnership with the renal system (J. Marks et al. 2013), and phosphate decreased distally. 4-hydroxyphenylacetic acid is a phenolic compound believed to be associated with microbial degradation of aromatic amino acids, and thus the increase along the GIT was not surprising. Garsin (2012) reviewed the function of ethanolamine in the GIT and specifically its relevance to enterohemorrhagic Escherichia coli O157:H7. In this study, it decreased along the GIT. Aspartic acid and serine are both amino acids that decreased along the GIT. Aminomalonate also generally decreased; this compound was identified by Van Buskirk et al. (1984) in proteins from Escherichia coli and in atherosclerotic plaque. Cytidine is a nucleoside component of RNA; the observed decrease may be due to degradation of dietary cytidine during normal digestion. While these seven compounds were consistently significantly different between sites using both PLS-DA and RFA, there are many compounds relevant to disease states or microbial metabolism that may not differentiate between sites within the canine GIT. The breadth of findings from an untargeted metabolomics approach in this study suggests we are only scratching the surface of the interactions between the microbiota and the host that may be metabolite mediated.

Complexity of the data prevented systematic investigation of all metabolite profile shapes across the four sites, but representative examples are shown in Figure 11 and application of more

objective methods to define these profile shapes would be warranted in future studies. The varied profiles observed are suggestive of underlying physiology dependent on the metabolite and location within the GIT. From the host perspective, the gut's primary function is absorption. However, this is not a linear process: many nutrients must be released from the whole-food matrix through enzymatic action (e.g., pancreatic enzymes or bacterial fermentation). As metabolites are liberated, they may spontaneously react further. The fate of each metabolite is somewhat unique, and changes in relative quantity at different locations of the GIT highlight this. The complexity of amino acid absorption has been reviewed and some species distinctions have been noted (Broer 2008). Studies specifically in the dog are scarce and focus on the jejunum as the site of absorption (Weber et al. 1977; Pytkowski and Michalowski 1977; Annegers 1969), yet the current study suggests several amino acids remain at high concentrations in the ileum. Additional studies are required to determine specific locations of amino acid transporters in the canine intestine.

The profile of pyruvate is also noteworthy. Only three other metabolites exhibited this profile where ileal abundances were significantly increased relative to duodenum and large intestine: xanthurenic acid, galactinol, and glucoheptulose. Proposing biochemical interpretations of this pattern would require imprudent conjecture, but it is worth highlighting that fecal concentrations of these compounds may be poor surrogate markers for concentrations elsewhere in the GIT.

2.4.4 Inter-omics correlations

Correlations between metabolites and bacterial families are not intended to suggest finding causality. Depletion of amino acids is likely partially coincidental to the relationships with the four bacterial families since the host actively absorbs amino acids. However, persistence of correlation across four sites within the GIT suggests the possibility of a stronger relationship, and Clostridia have long been appreciated as fermenters of amino acids (Mead 1971). The two metabolites that

showed strong positive correlations with bacterial families are closely related isomers, and are purported microbial degradation products of proanthocyanidins (Ward et al. 2004; Rios et al. 2003). Across samples, both bacterial families and both metabolites followed the same trajectory; thus it is interesting that the correlations (based on each individual sample) suggest more specific associations of 3-(3-hydroxyphenyl)propionic acid with Peptococcaceae and 3-(4-hydroxyphenyl)propionic acid with Veillonellaceae. Additional studies in vitro are required to determine if this correlation has relevance beyond coincidence.

Relationships between the gastrointestinal microbiota and metabolome in humans and animal models are also being explored in other labs (H. Lin et al. 2016; McHardy et al. 2013; Di Cagno et al. 2011; Sridharan et al. 2014), but rarely do these studies investigate multiple sites within the GIT. In comparison to single-site studies, such as looking at microbiota-metabolome correlations only within feces, the data in the present study demonstrated correlation across a greater breadth of environments.

2.4.5 Limitations

Samples were collected within three hours post mortem and it is unclear how rapidly the active biochemistry of the metabolites and the ecology of the microbiome might change after death. All six subjects were apparently healthy but the unavailability of complete medical histories leaves the possibility of antibiotic usage or other medications that could have affected the results. Furthermore, samples from only six dogs, all of hound-type breeding and with similar diet history, are unlikely to represent the entire breadth of diversity of the canine population. The influence of composition and timing of the last meal, as well as complete dietary history, should be considered in future studies exploring variation along the GIT. A naturally-passed fecal sample from each subject would also more directly allow correlation of fecal results to corresponding sites along the GIT. With regard to collection of samples, in the dog, the duodenum and ileum are somewhat

collapsed in vivo, so all contents from these sites are in close proximity to the mucosal surface. However, a distinction of mucosal and luminal sampling may be more relevant with regard to colonic and rectal contents; for the purposes of this study, we attempted to collect material representative of a transverse section, incorporating mid-luminal material as well as material adjacent to the mucosal surface, but the potential variation of these biogeographical regions is worth considering for future studies. Percent dry matter is known to vary in different samples of the GIT, and the decision to compare samples on the basis of wet-weight or dry-matter deserves careful thought. It may be considered that increased water content of a sample represents a physiological dilution of the concentration of metabolites within a sample (which has an effect on the biochemical kinetics), and therefore samples should be compared on the basis of wet weight. However, as water content can be highly variable and the dilution effect may be considered confounding (e.g., if comparing diarrheic to healthy fecal samples), the argument to normalize by dry matter (or some other method) is equally valid. Both approaches may introduce an inherent bias. Specifically for this study, none of the samples were overtly liquid upon collection, but clearly this is a subjective assessment and future studies should consider determination of dry matter content of different sites.

2.5 CONCLUSIONS

The data analyses in this work have explored two distinct but related data sets, 16S rRNA sequencing and metabolomics, from multiple angles. The application of PICRUSt allowed inferences of the predicted community functional potential, yielding an additional data set of KEGG Orthologs but which is completely dependent on the 16S rRNA sequencing results. Each of these data sets taken by itself provided descriptive information about the four sampled sites of the canine GIT, and subsequent inter-omics analysis demonstrated a potential approach to explore

the relationships between the data sets. To our knowledge, this is the first study of the metabolome in healthy dogs that included small and large intestinal sampling sites. Since a majority of microbiome studies use feces to represent the gut microbiome, it is important to elucidate and appreciate differences and similarities of the microbiota and metabolome along the GIT. Additional studies are required to elucidate the functional capability of the microbiota in vivo, explore the effect of dysbiosis on those functional capabilities, and improve our understanding of metabolites as potentially highly bioactive materials.

3. VARIATION OF CONCENTRATIONS OF STEROLS AND FATTY ACIDS ALONG THE HEALTHY CANINE GASTROINTESTINAL TRACT

OVERVIEW

As a brief addendum to the previous section, and to provide context for upcoming sections about the concentrations of fatty acids and sterols in various diseases, this section reports the quantitative results from analysis of the same samples as those used in Section 2, using the targeted assay described in Section 6.

3.1 INTRODUCTION

A critical part of interpreting data is understanding normal physiology. As described in Section 2, metabolites may exhibit different patterns of concentrations within different segments along the GIT. Lipid metabolism is a tremendously broad and complex topic, and understanding the mechanisms of absorption of lipids and their transport throughout the body are crucial for interpreting alterations in lipid metabolism during various disease states. In an effort to understand a small part of canine lipid metabolism and provide a framework for interpreting fecal results in the context of gastrointestinal diseases, the concentrations of fatty acids and sterols were determined in healthy dogs in duodenal, ileal, colonic, and rectal contents (n=6 dogs), as well as gall bladder content (n=8 dogs).

3.2 MATERIALS AND METHODS

Samples were collected as described in 2.2. The samples from duodenum, ileum, colon, and rectum are the same samples from the same animals reported in Section 2. However, bile samples were taken from an additional eight dogs at a different time. All samples were prepared and analyzed by GC-MS using the assay described in Section 6. Because the bile samples were

collected from an entirely distinct group of dogs, statistics were performed without consideration of dependence of samples (i.e., each sample site was treated as an independent group rather than repeated measures from individuals), using Kruskal-Wallis tests to determine which metabolites were significantly altered after a Benjamini-Hochberg correction for multiple comparisons, followed by a Dunn post-test to identify significant pairwise comparisons. Statistical significance was set at $p<0.05$.

3.3 RESULTS

With the exception of gondoic acid, all quantified metabolites exhibited a significant change in concentration in at least one site-pair comparison. The medians and ranges of concentrations of compounds in μg/mg lyophilized sample are presented in Table 7. Figure 12 shows the fatty acid concentrations of individual samples graphically, along with the sum of quantified fatty acids in each sample. Figure 13 shows the corresponding data for sterols, along with the total quantified sterols and the ratio of phytosterols to zoosterols.

Table 7. Concentrations of fatty acids and sterols in contents of the gastrointestinal tract. Superscripts identify which comparisons are statistically significantly different.

	Bile		Duodenum		Ileum		Colon		Rectum			
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range		
Compound	(µg/mg)		(µg/mg)		(µg/mg)		(µg/mg)		(µg/mg)		*p*-value	*q*-value
Fatty Acids												
α-linolenic acid	0.407 [a,c]	[0.274-1.246]	0.188 [b]	[0.155-0.571]	0.159 [b]	[0.091-0.663]	0.551 [c]	[0.333-0.768]	0.619 [c]	[0.468-0.849]	0.0049	0.0051
arachidonic acid	25.30 [a]	[20.69-29.7]	15.18 [b]	[12.81-20.46]	5.59 [c]	[3.13-17.79]	0.95 [d]	[0.61-9.99]	0.71 [d]	[0.27-1.34]	0.0000	0.0001
behenic acid	0.151 [a]	[0.122-0.213]	0.287 [b]	[0.214-0.311]	0.385 [c]	[0.304-0.411]	0.415 [d]	[0.392-0.653]	0.340 [c,d]	[0.322-0.477]	0.0000	0.0001
cis-vaccenic acid	5.38 [a]	[3.66-7.24]	1.53 [b]	[1.28-1.82]	0.94 [b,c]	[0.47-2.07]	0.92 [b,c]	[0.35-2.63]	0.99 [c]	[0.69-1.05]	0.0002	0.0003
erucic acid	0.019 [a]	[0.001-0.03]	0.044 [b]	[0.033-0.049]	0.036	[0.015-0.092]	0.070 [c]	[0.043-0.159]	0.040 [b,d]	[0.03-0.067]	0.0015	0.0017
gondoic acid	0.182	[0.149-0.262]	0.173	[0.144-0.2]	0.165	[0.084-0.239]	0.167	[0.097-0.228]	0.164	[0.142-0.252]	0.8197	0.8197
linoleic acid	17.96 [a]	[13.45-26.42]	13.51 [b]	[12.23-14.21]	5.78 [c]	[3.21-11.15]	4.03 [c]	[2.54-7.88]	4.13 [c]	[2.78-4.75]	0.0001	0.0001
nervonic acid	0.192 [a]	[0.135-0.384]	0.359 [b]	[0.315-0.388]	0.476 [c]	[0.332-0.637]	0.333 [b,c]	[0.295-0.715]	0.224 [a,d]	[0.169-0.301]	0.0004	0.0005
oleic acid	10.38 [a]	[8.3-17.7]	4.99 [b]	[4.24-6.13]	3.06 [b,c]	[1.62-6.57]	3.71 [c]	[2.07-4.78]	3.74 [c]	[2.55-4.47]	0.0003	0.0004
palmitic acid	16.24 [a]	[13.55-26.96]	6.43 [b]	[5.54-7.37]	5.14 [b,c]	[2.91-8.46]	4.27 [b,c]	[2.5-8.05]	4.16 [c]	[2.76-4.94]	0.0003	0.0004
stearic acid	13.20 [a]	[11.4-18.98]	11.89 [a]	[10.22-15.2]	6.19 [b]	[5.1-11.22]	3.41 [c]	[1.73-10.71]	3.29 [c]	[1.77-3.91]	0.0000	0.0001
Sterols												
β-sitosterol	0.111 [a]	[0.068-0.208]	0.089 [a]	[0.049-0.214]	0.728 [b]	[0.097-2.498]	4.443 [c]	[3.664-7.952]	4.250 [c]	[3.765-5.653]	0.0000	0.0001
brassicasterol	0.030 [a]	[0.015-0.065]	0.018 [b]	[0.014-0.021]	0.041 [a,c]	[0.021-0.055]	0.058 [c]	[0.038-0.084]	0.044 [a,c]	[0.029-0.068]	0.0022	0.0024
campesterol	0.312 [a]	[0.129-0.512]	0.247 [a]	[0.125-0.374]	1.356 [b]	[0.304-1.62]	2.953 [c]	[2.129-4.294]	2.190 [c]	[1.92-3.34]	0.0000	0.0001
cholestanol	0.091 [a]	[0.064-0.205]	0.072 [a]	[0.054-0.086]	0.205 [b]	[0.115-0.351]	0.330 [b]	[0.228-0.592]	0.173 [b]	[0.114-0.384]	0.0002	0.0003
cholesterol	1.83 [a]	[1.45-4.31]	15.20 [b]	[14.57-20.61]	15.23 [b]	[9.09-23.43]	7.89 [b]	[5.85-23.32]	3.98 [c]	[2.44-12.47]	0.0001	0.0001
coprostanol	0.015 [a]	[0.011-0.024]	0.011 [a]	[0.01-0.018]	0.047 [b]	[0.014-0.105]	0.138 [c]	[0.087-0.202]	0.130 [c]	[0.076-0.139]	0.0000	0.0001
fucosterol	0.016 [a]	[0.012-0.025]	0.015 [a]	[0.011-0.022]	0.054 [b]	[0.016-0.134]	0.243 [c]	[0.204-0.395]	0.217 [c]	[0.201-0.311]	0.0000	0.0001
lathosterol	0.026 [a]	[0.01-0.028]	0.039 [b]	[0.025-0.066]	0.141 [c]	[0.055-0.258]	0.162 [c,d]	[0.09-0.467]	0.051 [b,c]	[0.032-0.455]	0.0001	0.0002
sitostanol	0.008 [a]	[0.004-0.015]	0.005 [a]	[0.002-0.04]	0.055 [b]	[0.004-0.74]	1.200 [c]	[0.838-1.811]	1.152 [c]	[1.057-1.303]	0.0001	0.0001
stigmasterol	0.036 [a]	[0.028-0.055]	0.034 [a]	[0.026-0.067]	0.137 [b]	[0.037-0.588]	0.870 [c]	[0.716-1.286]	0.848 [c]	[0.8-0.988]	0.0000	0.0001

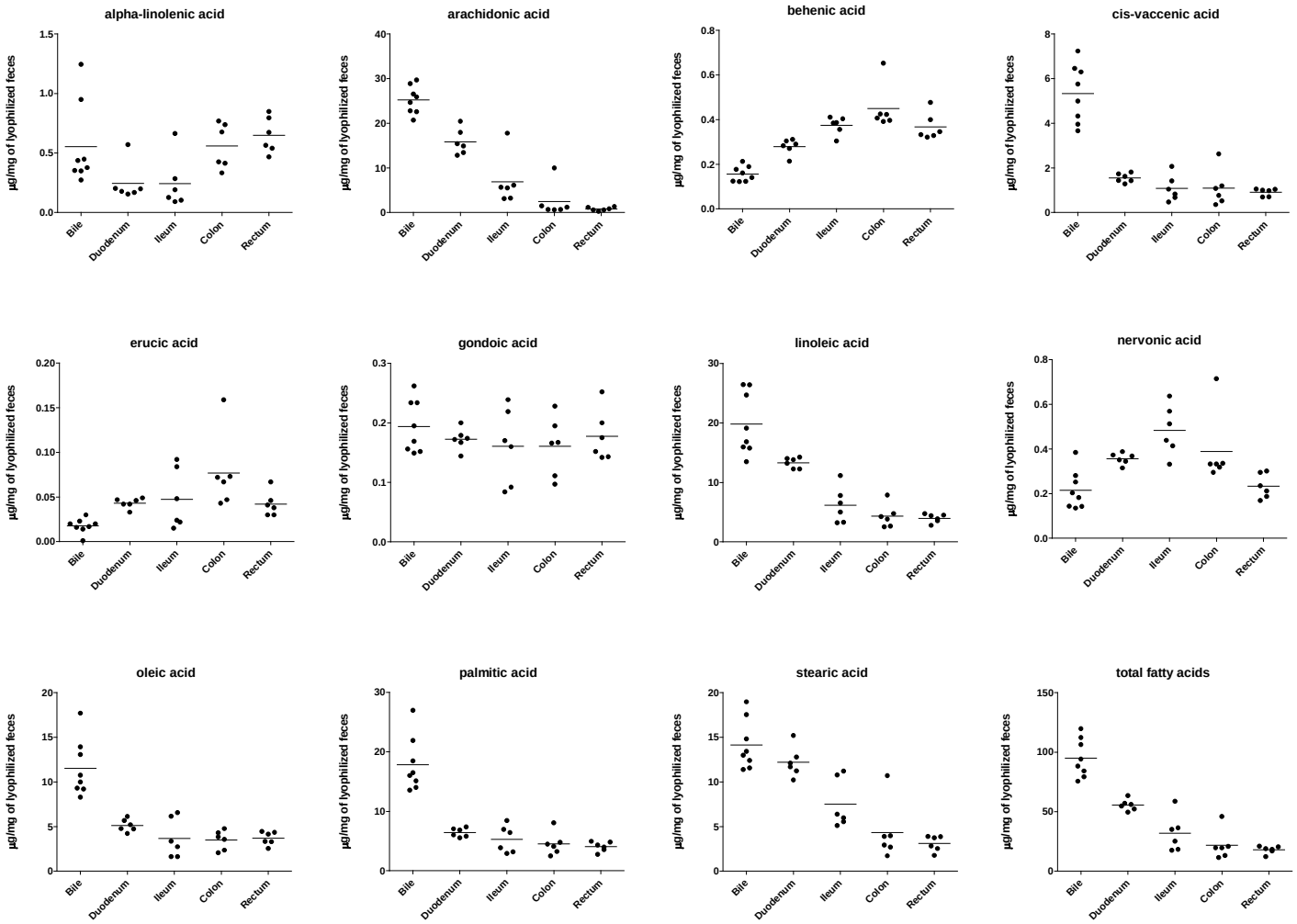


Figure 12. Concentrations of eleven fatty acids in bile and in samples taken at different sites along the GIT. The mean concentration is represented by a horizontal line.

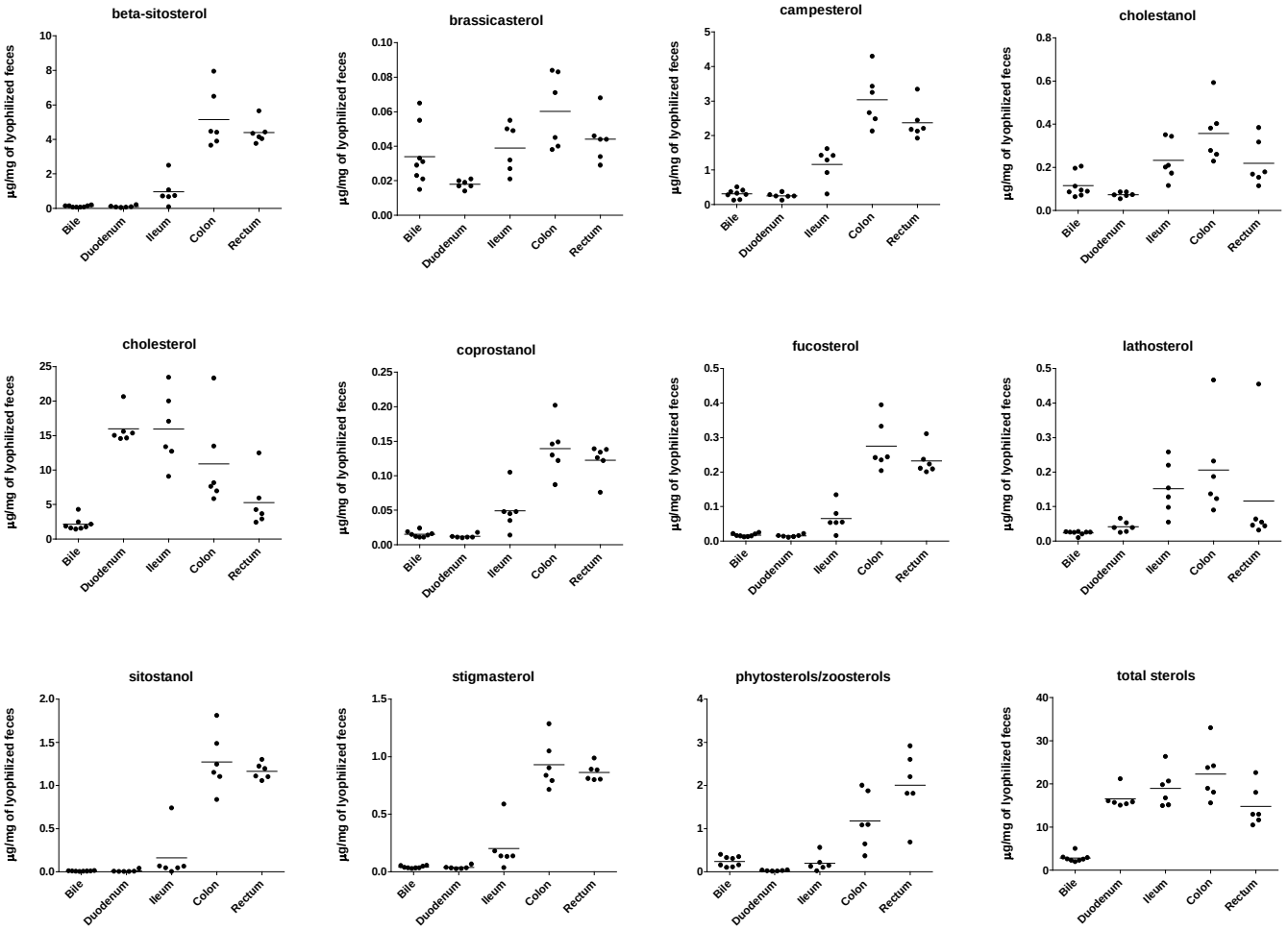


Figure 13. Concentrations of ten sterols in bile and in samples taken at different sites along the GIT. The total concentration of sterols and the ratio of phytosterols to zoosterols is also shown. The mean concentration is represented by a horizontal line.

3.4 DISCUSSION

The purpose of quantifying these compounds in samples from multiple sites within the GIT was largely to provide some context for interpretation of altered concentrations seen in feces of diseased animals, discussed in later sections. Based on the absorptive action of the GIT, it would be expected that concentrations of some metabolites would change. One may apply a concept that in a biological system, there are three causes for decreased concentrations of a given parameter (e.g., hematocrit on a complete blood count): destruction, loss, or failure to synthesize. Conversely, reasons for increased concentrations would be a decrease in physiological degradation or excretion, or increased synthesis. These guidelines are not as readily applied to concentrations in intestinal contents, where secretion by the host increases luminal concentration and absorption decreases it, but generally the changes in concentration at different sites of the GIT can be interpreted based on similar principles. Given the increasing concentrations of several of the phytosterols from duodenum to rectum (i.e., β-sitosterol, campesterol, fucosterol, sitostanol, and stigmasterol), the factors that contribute to this increase must be explored. First, the quantity of material consumed on a dry matter basis is significantly greater than the quantity excreted as feces. Actual values from the literature are not readily available, but one article reported fecal outputs (i.e., wet-weight) from humans in Westernized populations as only 80-120 g/day (Cummings et al. 1992). Specifics of the mass of dietary intake vary tremendously and are highly dependent on water content of the diet, especially for the diverse diets of humans, but based on data from the National Health and Nutrition Examination Survey (NHANES) the average American may consume quantities on the order of 2.5 kilograms of food per day, a 20-fold factor relative to the 0.12 kg fecal output. Even considering these values are for humans and without correcting for water content, it should be apparent that among healthy individuals, any compound that has zero

net absorption and excretion would show increased concentrations more distally because of the decreasing total mass of the digesta, which should be very important for interpretation of all results from samples taken at different segments along the GIT. However, while this may alter the interpretation of increasing concentrations to instead mean no net absorption, it does not diminish the importance to biochemical equilibrium dynamics caused by concentration gradients, nor does it reduce the significance of altered concentrations when comparing two groups of same-site samples (i.e., feces from diseased dogs compared to healthy dogs).

Specifically with regard to phytosterols, there is a second mechanism that may contribute to the apparent increase of concentrations observed in healthy animals. As described in Section 1.6, the NPC1L1 transporter is preferential for cholesterol but does not completely exclude other sterols (Tang et al. 2009). Selectivity for absorption of cholesterol is secondarily accomplished via ABCG5 and ABCG8 transporters, which preferentially expel phytosterols rather than cholesterol. Therefore, over the length of the intestines, it would make sense that the concentrations of phytosterols are further increased by the selective expulsion of phytosterols from the enterocytes with concurrent absorption of cholesterol, as supported by the decreasing luminal concentrations of cholesterol from duodenum to rectum.

The composition of bile includes water, bile salts, cholesterol, and lecithin. Lecithin is usually a mix of phospholipids, whether phosphatidylcholine, phosphatidylethanolamine, or a glycerophospholipid. As discussed in Section 1.5, phospholipids usually have a saturated fatty acid at carbon 1 and an unsaturated fatty acid at carbon 2. Interestingly, the choline in phosphatidyl choline has been implicated in cardiovascular disease in humans, in a microbiota-mediated pathway involving metabolism of choline to trimethylamine oxide (TMAO).

Due to the concentrating effect of general absorption and decrease of luminal contents, the magnitude of decrease of compounds along the GIT may in fact be underestimated.

Cholesterol, for example, appears to decrease from 15 μg/mg in the duodenum to 5 μg/mg in the rectum. If the 100-fold overall dry-matter mass decrease is mathematically reversed for the purpose of demonstrating this concept, the rectal contents would instead be 5 μg per 100 mg, or 0.05 μg/mg, which is a 300-fold decrease in concentration. If equilibrium dynamics are then considered, it is interesting that additional extraction of a compound at such low concentrations would be thermodynamically disfavored, but by concentrating the compounds in the lumen, there is increased opportunity for both passive and active transport across enterocyte membranes.

3.5 CONCLUSIONS

Lipid metabolism is a complicated and multifaceted topic. While some lipids (e.g., cholesterol) clearly undergo net absorption, it is more difficult to interpret the metabolites that appeared to increase in concentration on a μg/mg basis (i.e., phytosterols) since the absorptive processes in the GI tract have a concentrating effect on the luminal contents. Nonetheless, understanding how fecal concentrations of sterols and fatty acids relate to concentrations in more proximal segments of the gastrointestinal tract provides an important context for interpretation of fecal results when comparing diseased dogs to healthy control dogs.

4. BIOMOLECULAR PATHWAYS OF BACTERIA IN THE DUODENUM OF DOGS WITH IDIOPATHIC INFLAMMATORY BOWEL DISEASE

OVERVIEW

The present understanding of canine idiopathic inflammatory bowel disease (IBD) assigns a significant role to the interplay between the gastrointestinal microbiota, the immune system, and host genetic factors in the pathogenesis of the disease. Sequencing of the 16S rRNA gene has shown significant alterations in microbial groups among dogs with IBD compared to healthy control dogs, but the functional effect of the resident microbiota in health and disease has not been elucidated. The aim of this study was to utilize a computational approach to explore possible alterations in microbial biomolecular pathways between dogs with idiopathic IBD and healthy control dogs.

Previously published 16S rRNA gene sequencing data describing alterations in the microbiota of duodenal biopsies from dogs with moderate to severe IBD (n=14) and healthy control dogs (n=6) were used for this study. The data were analyzed using the bioinformatics software PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) to predict the functional capabilities of bacteria within each sample. The relative abundance of genes associated with a given pathway may indicate an increased metabolic capacity of the enteric microbiota with regard to that pathway. Inferences of the functional gene content were grouped by function according to the three-level KO (Kyoto Encyclopedia of Genes and Genomes Ortholog) hierarchy and compared using Linear Discriminant Analysis (LDA) through LEfSe (LDA Effect Size) with the significance threshold set to an LDA score of log 2.5.

Among pathway categories, lipid metabolism was increased in dogs with IBD (LDA=3.4), while pathways associated with carbohydrate, nucleotide, and energy metabolism were relatively

decreased when compared to healthy control dogs (LDA=3.7, 3.5, and 3.5; respectively). Pathways categorized into translation or replication and repair, which are subcategories of genetic information processing, were decreased in abundance among dogs with IBD (LDA=3.6 and 3.8, respectively) while the category of environmental information processing was more abundant in the dogs with IBD compared to healthy control dogs (LDA=4.0). At the individual KO level, the iron complex outer membrane receptor protein was significantly increased in dogs with IBD (LDA=3.2), along with two KOs associated with the NitT/TauT family transport system (LDA=2.7, 2.5), which is involved in osmoprotection. The abundances of three KOs associated with the multiple sugar transport system were decreased in dogs with IBD (LDA=3.0, 2.9, and 2.7). The abundance of the KO corresponding to 6-phosphofructokinase 1 was also decreased relative to the healthy control dogs (LDA=2.5).

These predictions of the functional metagenomic milieu provide insights into the changes occurring in the duodenum in dogs with idiopathic IBD. Future studies are warranted to evaluate these pathways and related metabolites for potential diagnostic and/or therapeutic targets.

4.1 INTRODUCTION

Idiopathic inflammatory bowel disease (IBD) is an important disease in dogs and has been associated with gastrointestinal dysbiosis (Suchodolski et al. 2010; Minamoto et al. 2015; Vazquez-Baeza et al. 2016). The functional effects of the shift in microbial populations, including production of metabolites and interaction with host systems, have not yet been well-characterized. In addition, samples used to characterize the microbiota associated with IBD are typically naturally passed feces, while the histologic evidence of inflammatory lesions is often observed in the duodenum. There is a clear need for improved understanding of the changes occurring at the sites

within the GI tract that are affected by the disease, which would require endoscopy or laparotomy to obtain samples from the patients.

Upon obtaining samples from sites of disease, specific experiments and analyses must be chosen from an array of available tests. Based on the presumed interplay between host and microbiota implicated in disease, it is also relevant to consider which organisms are desirable to target in the analysis. Tissue biopsies are primarily comprised of host cells, but the mucosa is known to harbor bacteria as well, though much more sparsely in the proximal than the distal GI tract. Experimentally determining metabolic activity through transcriptomics is expensive, and performed on biopsies primarily captures the effects of gene regulation on the part of the host, not the microbiota. Whole-genome sequencing (WGS) on a sample of the microbiota (excluding host cells) is also costly, but in theory would characterize the microbial metagenome. 16S rRNA sequencing is quite affordable and is frequently used to determine the composition of the bacterial microbiota in a sample. The resulting data set does not directly speak to the function (actual or potential) of the microbiota. However, computational methods have been shown to effectively predict a more complete microbial composition from 16S rRNA sequencing data, that is, beyond what can be directly observed (Langille et al. 2013). The identities of the predicted microbiota can then be used to predict the metagenomic composition of the microbial community as a whole using public databases. Finally, the predicted metagenome can be translated into gene sequences associated with specific proteins, where the gene function may be known to play a role in one or more metabolic or structural pathways, using a database such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2016). The pathways can subsequently be grouped into categories to observe more general themes. In other words, relative changes in the abundances of pathways are suggestive of up- or down-regulation of biosynthesis or degradation of specific metabolites or host-microbe interaction signaling molecules. Importantly, since the data for the

pathways originates exclusively as 16S rRNA sequencing data, the predicted metagenome and associated pathways must be associated specifically with the bacterial microbiota, not the host metagenome. Although the method involves predictive steps *in silico*, it is an interesting tool for viewing the microbial composition as a functional organ, and that comparing samples from diseased and healthy dogs may help elucidate how the disease state shapes the metabolic capacity of the microbiota. The objective of this study was to identify significant functional differences between the microbiota of dogs with idiopathic IBD and healthy control dogs, as evidenced by predicted alterations in bacterial biomolecular pathways, using a computational approach. Previously published data sets related to this study include host gene expression data (Wilke et al. 2012) and mucosal microbiota composition (Suchodolski et al. 2012a).

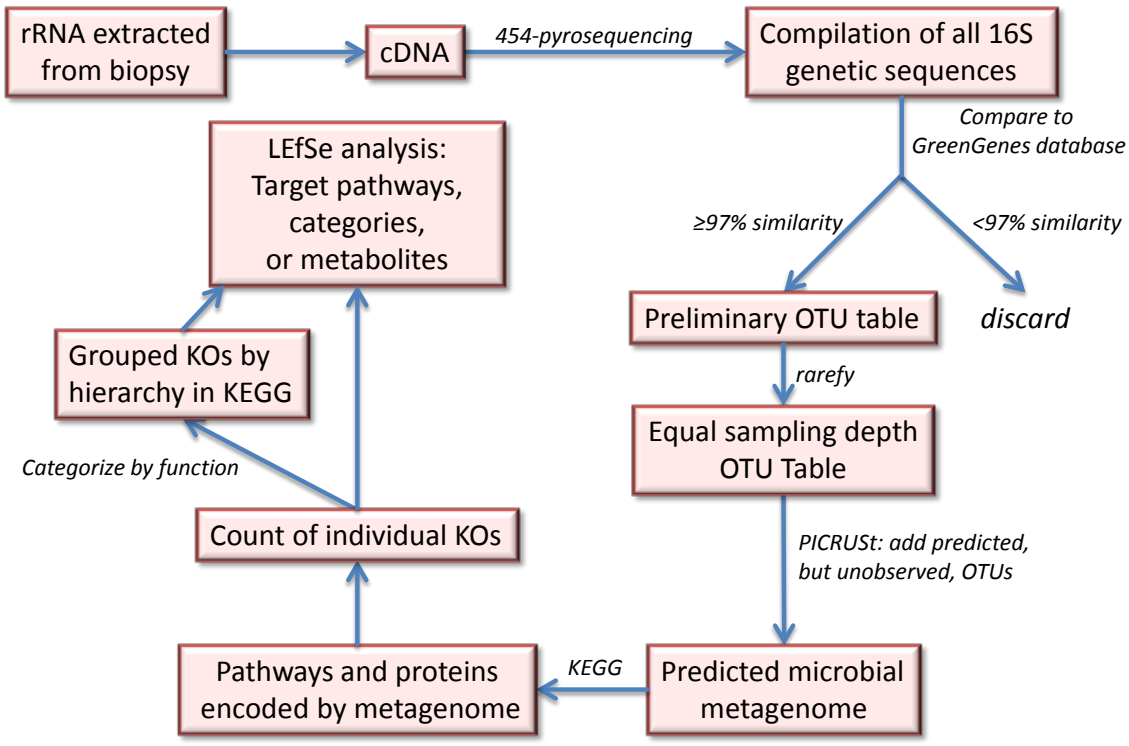


Figure 14. PICRUSt project workflow. OTU: operational taxonomic unit; KEGG: Kyoto encyclopedia of genes and genomes; KO: KEGG ortholog

4.2 MATERIALS AND METHODS

The overall flow of the study is depicted in Figure 14.

4.2.1 Sequencing data

The associated 16S rRNA sequencing data obtained from duodenal mucosal biopsies from dogs with IBD (severe, *n*=7; moderate, *n*=7) and healthy control dogs (*n*=6) has previously been published (Suchodolski et al. 2012a). In brief, RNA was extracted from endoscopically-obtained biopsies and transcribed into cDNA according to kit instructions. 454-pyrosequencing of the 16S rRNA gene resulted in raw data that was initially processed using QIIME version 1.4.0, and chimera removal was performed using B2C2 to yield the data set for the current study (Caporaso et al. 2010b; Gontcharova et al. 2010).

4.2.2 Data analysis

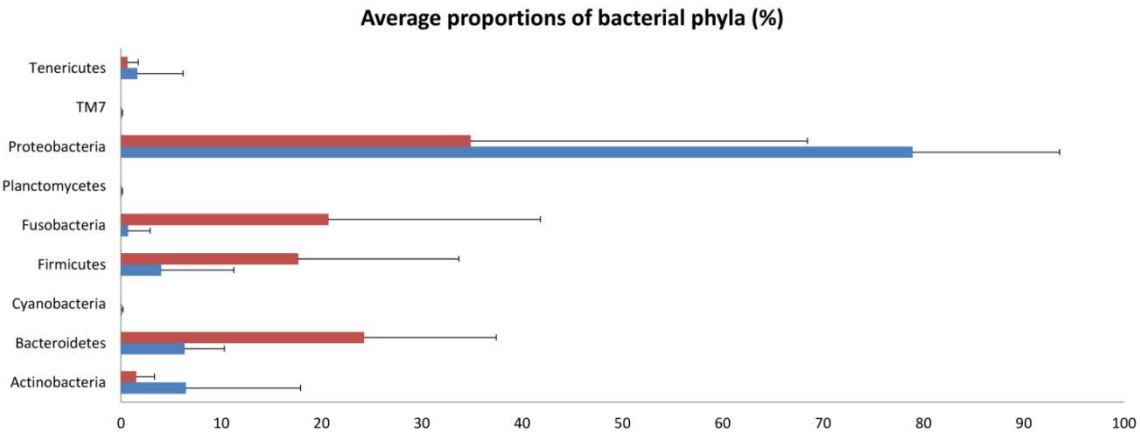
The GreenGenes database version 13.5 was used as the reference for picking Operational Taxonomic Units (OTUs) of ≥97% match to known OTUs (DeSantis et al. 2006). This closed-reference strategy is appropriate for matching 16S rRNA sequencing data to appropriate taxonomy and applying established phylogenetic relationships in downstream analysis. Although the closed-reference strategy does exclude OTUs that are not in the database, and version 13.5 is not the most up to date version, it is the only version that can be interfaced with the bioinformatics software PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) at the time of this writing. Data processing steps were carried out within the software PICRUSt using scripts. Unequal sampling depth was accounted for by random selection of 683 sequences, the minimum number in any single sample. The OTU table was normalized by copy number, the metagenome predicted, and assignment of predicted gene sequences was made to one or more KEGG Orthologs (KO; Kyoto Encyclopedia of Genes and Genomes) (Langille et al. 2013;

Kanehisa et al. 2014; Kanehisa and Goto 2000). KOs were categorized according to the three-level KO hierarchy to look for broader trends. An individual KO, representing a single gene sequence that may encode a structural protein or enzyme, in whole or in part, may be included in more than one category if merited by the function of the protein in the organism.

Pathway abundances (categorized and uncategorized) were compared between the predicted metagenome of dogs with IBD and healthy control dogs using Linear Discriminant Analysis (LDA) and the online data analysis package LEfSe (LDA Effect Size), where the significance threshold was set to log 2.5 (Segata et al. 2011).

4.3 RESULTS

Previously published results of average proportion of bacterial phyla and the clustering in accordance with clinical severity of disease are shown in Figure 15 and Figure 16.



	Actinobacteria	Bacteroidetes	Cyanobacteria	Firmicutes	Fusobacteria	Planctomycetes	Proteobacteria	TM7	Tenericutes
Control	1.5	24.2	0.0	17.7	20.7	0.0	34.9	0.0	0.7
IBD	6.5	6.3	0.1	4.0	0.7	0.1	78.9	0.1	1.6

Figure 15. Average proportion of phyla in healthy control dogs and dogs with IBD. Values are the percent of total 16S rRNA sequences identified. Previously published work (Suchodolski et al. 2012a).

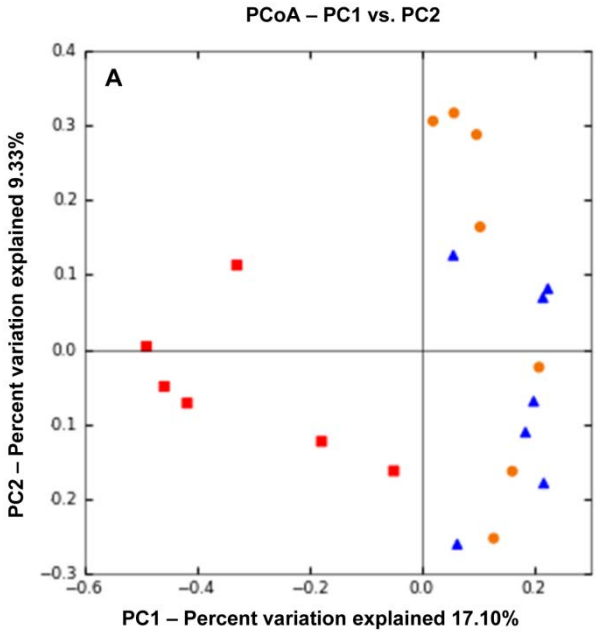


Figure 16. Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16S rRNA genes based on clinical severity of disease. Each spot represents the microbiome of a single sample and distances between spots correspond to similarity of the microbiome. Healthy control dogs (red squares) clustered separately from dogs with disease (blue = moderate; orange = severe). Previously published work (Suchodolski et al. 2012a).

4.3.1 Categorized by function according to KEGG hierarchy

The cladogram in Figure 17 displays increased and decreased abundances of pathways within hierarchies. The structure of the cladogram is determined by the relationships of subcategories to categories; for example, environmental information processing is a level 1 category that was significantly increased in dogs with IBD. The level 1 alteration was likely driven by the level 3 subcategory of signal transduction, which in turn likely drove the significance of the two component system category, from level 2. Within a hierarchical level, the cladogram also attempts to co-locate more similar functions together. For example, DNA repair and recombination proteins are adjacent to DNA replication proteins, while the category for ribosome is somewhat farther away, though all three subcategories are in the same level 1 group of genetic information

processing. The data can be visualized independently of this clustering strategy in the bar chart in Figure 18, which simply displays the LDA score for each category. Since the cladogram displays all significant features but does not display the LDA score, the two figures are complementary in terms of how they visualize the data.

Among some of the findings of interest, the total predicted abundance of genes associated with lipid metabolism is increased in the metagenome of dogs with IBD (LDA=3.4). Pathways categorized as energy metabolism or carbohydrate metabolism were decreased (LDA=3.5 and 3.7, respectively), with significant subcategories of methane metabolism (LDA=3.1), amino sugar and nucleotide sugar metabolism (LDA=3.2), and starch and sucrose metabolism (LDA=3.2). As seen in the cladogram, pathways associated with the two component system, nested within the signal transduction subcategory of environmental information processing, are increased in abundance among dogs with IBD (LDA=3.4, 3.4, and 4.0, respectively). Genetic information processing pathways, through subcategories of both translation and replication and repair, are relatively decreased in abundance among dogs with IBD (LDA=4.0, 3.6, and 3.8). Importantly, significant features can be masked by the nature of the hierarchical structure of the categories/subcategories. If an enzyme responsible for microbial lipid transport were decreased in abundance, while a different enzyme responsible for lipid degradation were increased, there may be no net effect to the abundance of the category of lipid metabolism. For this reason, it is also important to explore the data as individual KOs, irrespective of their categorization into the hierarchical structure.

4.3.2 Individual KEGG orthologous genes

When the data were analyzed as individual KOs, 23 exceeded the significance threshold of LDA=log 2.5 (Table 8). For illustrative purposes, an example of the distribution across all of the samples of a particular KO is shown in Figure 19, in this case showing K02014, iron complex outer membrane receptor protein. This KO was significantly increased in dogs with IBD

(LDA=3.2). Other uncategorized KO findings included two KOs associated with the NitT/TauT family transport system, which is involved in osmoprotection through transport of nitrate and taurine, and were significantly increased in the microbiome of dogs with IBD (LDA=2.7, 2.5). The abundances of three KOs associated with the multiple sugar transport system were decreased in dogs with IBD (LDA=3.0, 2.9, and 2.7). The abundance of the KO corresponding to the enzyme 6-phosphofructokinase 1 was also decreased relative to the healthy dogs (LDA=2.5).

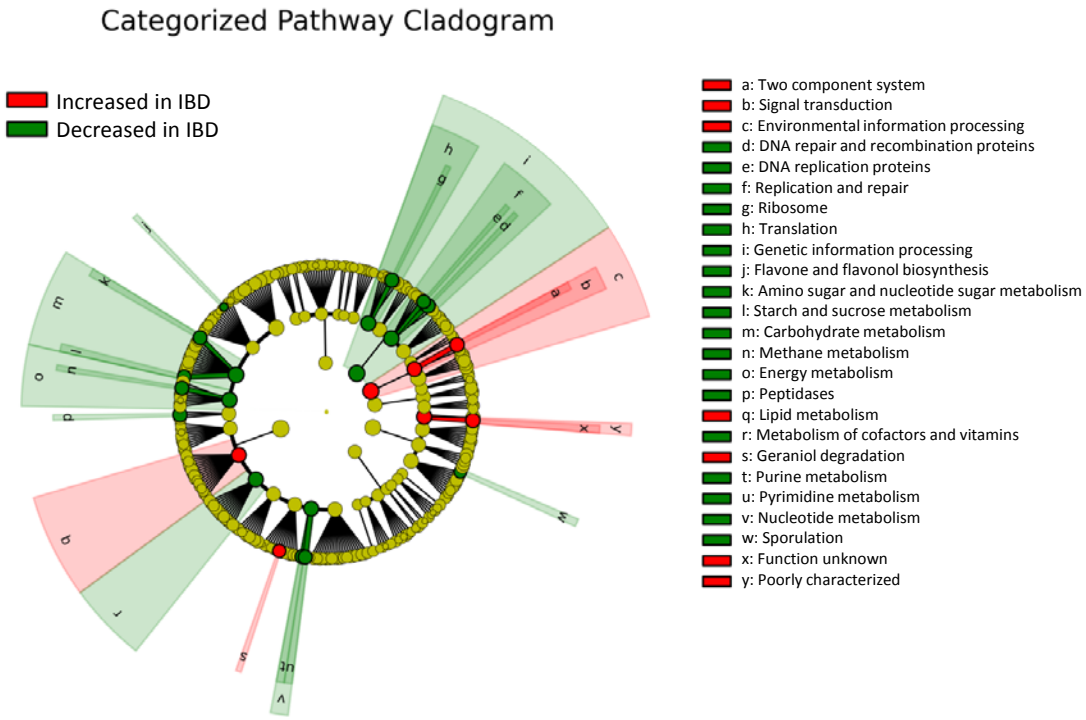


Figure 17. Cladogram of pathways organized by KEGG hierarchy. Relative changes in abundances of pathway categories may allow inference of selective pressure within the gastrointestinal tract or suggest components playing a role in the pathophysiology of IBD. Individual pathways are not shown as they may be involved in more than one category.

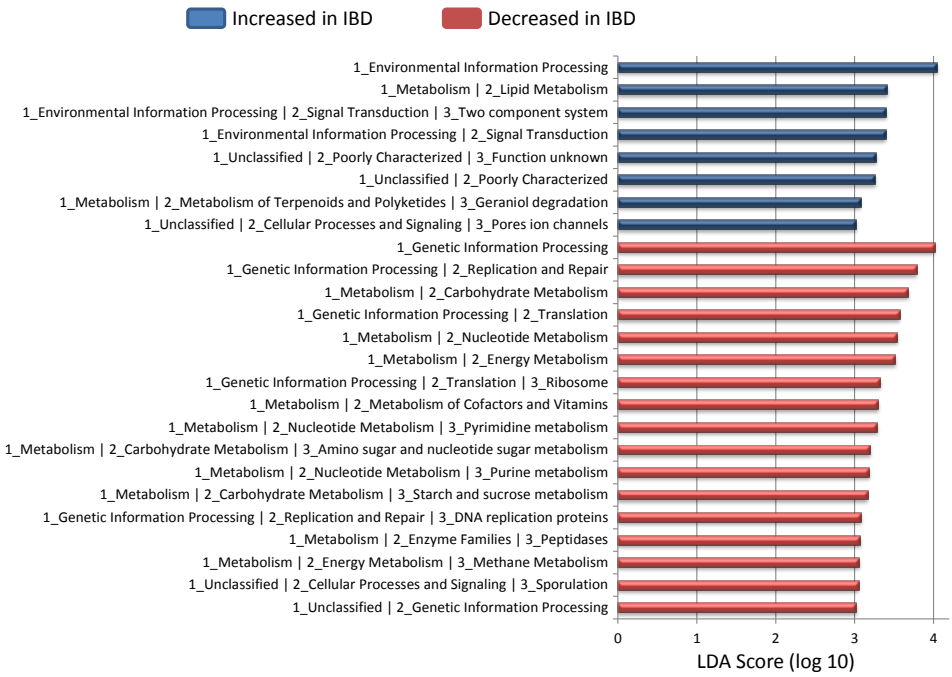


Figure 18. Linear discriminant analysis effect size (LEFSe) of categories. LEfSe identifies significant differences between groups using nonparametric factorial Kruskal-Wallis sum-rank test, then uses Linear Discriminant Analysis to estimate the effect size of each different feature.

Table 8. Individual KEGG orthologs that were significantly altered in dogs with IBD.

Change in IBD	LDA score (log)	KEGG ID: Description
decreased	2.98	K02025: multiple sugar transport system permease protein
	2.91	K02026: multiple sugar transport system permease protein
	2.87	K06147: ATP-binding cassette, subfamily B, bacterial
	2.74	K02027: multiple sugar transport system substrate-binding protein
	2.69	K01190: beta-galactosidase
	2.68	K07024: K07024 (hypothetical protein; hydrolase)
	2.65	K03497: chromosome partitioning protein, ParB family
	2.64	K03530: DNA-binding protein HU-beta
	2.59	K07133: K07133
	2.59	K05349: beta-glucosidase
	2.57	K03169: DNA topoisomerase III
	2.56	K01990: ABC-2 type transport system ATP-binding protein
	2.53	K00850: 6-phosphofructokinase 1
	2.53	K03091: RNA polymerase sporulation-specific sigma factor
	2.52	K06180: 23S rRNA pseudouridine1911/1915/1917 synthase
	2.51	K07240: chromate transporter
increased	3.18	K02014: iron complex outer membrane receptor protein
	2.69	K02051: NitT/TauT family transport system substrate-binding protein
	2.68	K02029: polar amino acid transport system permease protein
	2.60	K03293: amino acid transporter, AAT family
	2.55	K00140: malonate-semialdehyde dehydrogenase (acetylating) / methylmalonate-semialdehyde dehydrogenase
	2.51	K02050: NitT/TauT family transport system permease protein
	2.51	K06911: K06911

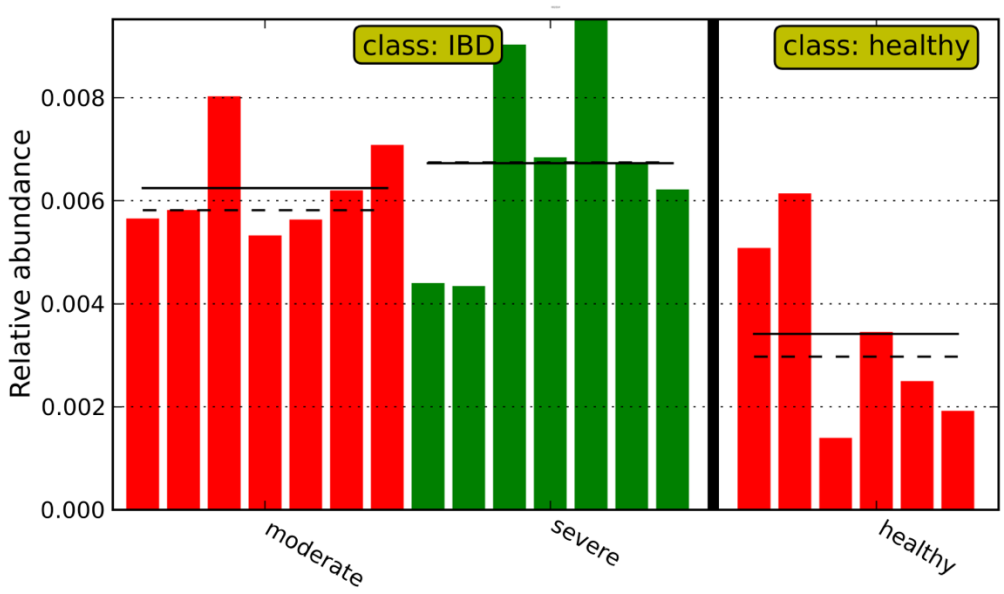


Figure 19. KO abundance of iron complex outer membrane receptor protein. Each bar represents an individual sample; the samples from dogs with IBD are also grouped by disease severity. Solid and dashed lines indicate the subclass mean and median, respectively.

4.4 DISCUSSION

It has previously been shown in humans that the rectal mucosa-associated microbiota is a better indicator of disease than the fecal microbiota (Gevers et al. 2014), potentially indicating that the organisms more closely interfacing with the host immune system have greater impact on disease status. The sequencing data generated from duodenal mucosal biopsies in this study may thus be of greater relevance to disease status compared to that generated from canine feces. The *in silico* conversion of sequencing data to predicted metagenomic community potential is an interesting alternative approach to viewing the sequencing data from a functional perspective. It has been established that the breadth and diversity of metabolic potential of the gut microbiota greatly exceeds the metabolic functions encoded by the host genome. Furthermore, the microbiota

as a community represent a highly adaptive metabolic repertoire that when altered may play a role in either the onset or perpetuation of gastrointestinal diseases. This is reflected in the observation that a population shift of the gastrointestinal microbiota (dysbiosis) occurs among dogs with IBD, possibly reflecting selective pressure by the inflamed gastrointestinal environment and/or some other underlying facet of the etiopathogenesis of IBD.

The two component system pathways, which were significantly more abundant in the predicted bacterial metagenome of dogs with IBD, may suggest an instability of the gastrointestinal environment, necessitating enhanced sense-and-response capabilities among resident microbiota. Concurrently, the significant decrease in genetic information processing pathways and subcategories could be suggestive of a microbial composition that is less prepared for replication, though assigning such specific functions to such broad categories is likely an overstatement of the data.

Also observed were relative increases of lipid metabolism pathways and decreased carbohydrate metabolism. This may suggest a role of lipid-associated metabolites, including sterols, fatty acids, or bile acids, or could reflect an effect of the higher median (and range) fat content of diets fed to study dogs with IBD compared to the standard ration fed to all control dogs (4.1 (2.9-5.0) versus 2.5 g of fat/100 kcal ME). Additionally, the importance of the intestinal mucosa in the assimilation of lipids has been briefly discussed in Section 1.1, and altered function of the mucosa in a diseased state (due to inflammation) could be implicated in an altered composition of lipids in the lumen, which in turn may exert a selective pressure on the microbiota and thus drive changes in the metabolic capacity represented by the microbiota in dogs with IBD compared to healthy control dogs.

Among individual KOs, the increased abundance of K02014 (iron complex outer membrane receptor protein) may occur with gastrointestinal bleeding, which increases the

concentration of iron in the lumen. It is also interesting to point out that there were several KOs listed that had limited or no description: K07133, K06911, and K07024. In these cases, the predicted gene sequence was recognized as encoding a relevant (that is, putatively functional) protein, but the function of said protein is unknown. However, the database of KOs is continuing to expand as new sequences are identified in a broader range of organisms. K07024 has since been further identified to be sucrose-6-phosphatase, an enzyme that plays a role in starch and sucrose metabolism. K06911 and K07133 remain uncharacterized proteins. To take complete advantage of the expanding databases, the components of PICRUSt will also require updating, as the vast range of metabolic functions attributed to the GI microbiota continues to expand.

4.5 CONCLUSIONS

Exploring how the abundances of metabolic functions or pathways changes in different disease states may help elucidate how bacterial species and their metabolites play a critical functional role in disease pathogenesis. The computational approach employed in this study allows reanalysis of existing data from a new perspective, highlighting differences at a functional level that might not have been observed when looking at the data purely based on bacterial identities. An in depth examination of these data compared to the host gene expression data (Wilke et al. 2012) might also yield interesting correlations, though methods to combine different dependent data sets in meaningful ways (that is, beyond purely mathematical correlations) remain relatively unproven. Further studies are needed to evaluate metabolites involved in the identified categories and pathways for their utility as diagnostic and/or therapeutic targets.

5. DISCOVERY OF POTENTIAL FECAL BIOMARKERS FOR CHRONIC ENTEROPATHY USING UNTARGETED METABOLOMICS

OVERVIEW

Intestinal dysbiosis in dogs is defined as a relative shift in composition of bacterial populations in the gastrointestinal tract (GIT) and has been associated with chronic enteropathy (CE). Our group has previously reported small intestinal or fecal dysbiosis and decreased species richness in canine spontaneous inflammatory bowel disease. However, due to functional redundancy between bacterial groups, determining the biochemical changes in the GIT requires direct analysis of the biomolecules that are present. The aim of this study was to evaluate differences in fecal metabolite profiles between dogs with chronic gastrointestinal inflammation and healthy control dogs.

Fecal samples were collected from dogs with histopathological evidence of gastrointestinal inflammation (n=15) and from healthy control dogs (n=15). The fecal metabolome, comprised of metabolic products from both the host and the microbiota, was assessed using an untargeted approach combining LC-MS and GC-MS platforms. Random Forest Analysis was used to rank differentiating power of metabolites, and the Mann-Whitney U-test followed by the Benjamini-Hochberg adjustment of p-values for multiple comparisons was used to test for statistical significance (q<0.05).

A total of 787 named biochemical compounds were identified. Of these, 145 were significantly altered after adjusting for multiple comparisons (q<0.05). Among major metabolic pathways, some metabolites associated with GIT redox homeostasis were altered, including significantly increased concentrations of precursors of glutathione: 5-oxoproline (q=0.004), cysteine (q=0.024), glycine (q=0.003), and γ-glutamyl amino acid derivatives of lysine,

phenylalanine, valine, and leucine (all $q \leq 0.015$). Primary bile acids were generally increased in dogs with disease (cholate, $q=0.013$; chenodeoxycholate, $q=0.055$), while secondary bile acids were generally decreased in dogs with chronic enteropathy (deoxycholate, $q=0.045$; lithocholate, $q=0.059$), suggesting impaired bacterial conversion from primary to secondary bile acids. Metabolites within the aromatic amino acid biosynthesis pathway exhibited some of the most significant alterations. The tryptophan metabolites indoleacetate and indolepropionate were significantly decreased in dogs with disease ($q=0.008$ and 0.030, respectively). 2-oxindole-3-acetate, an oxidative degradation product of indoleacetate, was 100-fold decreased in diseased animals ($q=0.001$) and was highly ranked for discriminating power.

This study demonstrates that feces are a rich sample matrix for biomolecules, representing both the host and microbial metabolite profiles. Several metabolites were identified that may be investigated as future biomarkers and may help elucidate the etiopathogenesis of canine chronic enteropathy.

5.1 INTRODUCTION

Chronic enteropathy (CE) is a complex group of diseases. It is characterized by clinical signs of gastrointestinal disease of at least three weeks of duration. The most frequent presenting complaint is diarrhea, but vomiting and weight loss are also commonly reported. For both CE in dogs and IBD in humans, changes in the gut microbiota have been strongly associated with the disease, although conclusive evidence is lacking as to whether this is a direct cause or effect, or simply part of the perpetuation of chronic abnormal immune activation. The microbiota are now considered by some to be an organ, inextricably linked to host health but having a somewhat independent physiology and can develop unique pathology. One means of pathology of the microbiota is an abnormal composition, or dysbiosis. A 454-pyrosequencing study in dogs based

on fecal 16S rRNA genes revealed decreased bacterial diversity, with increased Gammaproteobacteria and decreased Erysipelotrichia, Clostridia, and Bacteroidia in the fecal microbiota of dogs with IBD compared to healthy control dogs (Minamoto et al. 2015). The study also investigated the serum metabolite profiles of the dogs. Multivariate analyses suggested changes of the global serum metabolite composition, but few individual compounds were statistically significantly altered in the serum of dogs with IBD. Considering the location of lesions of IBD being the mucosa of the GI tract, and the capacity for control and correction that the host may have on the circulatory system, it would make sense that more profound changes might be observed by analyzing the composition of the digesta or feces. A study of a very large cohort of treatment-naïve pediatric Crohn's disease (CD) patients included samples from the ileum, rectum, and feces (Gevers et al. 2014). The dysbiosis they reported in CD was not dissimilar to that reported in dogs, with decreases observed in Bacteroidales, Clostridiales, Erysipelotrichaceae, though they found that the mucosa-associated rectal microbiota correlated better to disease status than the fecal microbiota. The study also employed PICRUSt to interpret the sequencing data as a predicted collective functional capacity, finding what the authors described as associations between disease severity and oxidative stress, benzoate metabolism, loss of basic biosynthesis, and a "switch toward pathobiont-like auxotrophy," implying a developing dependence on the host and concurrent change from symbiont to pathogen. However, PICRUSt is dependent on the 16S rRNA sequencing data, and is purely predictive of functional capacity but not actual metabolic activity. The actual biochemical composition of feces in disease versus health, shaped by both the metabolic effects of the host and the microbiota, could potentially highlight relevant alterations that are integral to disease status. The current study aim was to evaluate differences in fecal microbiota and metabolite compositions between dogs with chronic gastrointestinal inflammation and healthy control dogs.

5.2 MATERIALS AND METHODS

5.2.1 Study population and sampling

A subset of patients from an ongoing research project at the Gastrointestinal Laboratory (College of Veterinary Medicine, Texas A&M University, College Station, TX, USA) with a tentative diagnosis of chronic enteropathy and histopathological evidence of gastrointestinal inflammation and where surplus naturally-passed fecal samples were available (n=15) were retrospectively identified. Animals with reported immunomodulatory drug usage for treatment of the GI signs were excluded. A combination of banked and prospectively collected fecal samples from healthy control dogs (n=15) were also assembled.

5.2.2 DNA extraction and sequencing

Approximately 100 mg of each sample was taken for DNA extraction according to manufacturer's protocol (ZR Fecal DNA MiniPrep, Zymo Research, Irvine, CA, USA). Amplification and sequencing of the V4 variable region 16S rRNA gene was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA). Briefly, samples were barcoded and PCR primers 515F/806R were used in a 28-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, with final elongation at 72°C for 5 minutes. A DNA library was prepared according to the Illumina TruSeq DNA library preparation protocol. Sequencing was performed on a MiSeq (Illumina) following the manufacturer's guidelines. Sequence data were uploaded into the NCBI GenBank database under submission number SRP090443.

5.2.3 Analysis of sequences

Quantitative Insights Into Microbial Ecology (QIIME, v. 1.8) was used for processing and analysis of sequences (Caporaso et al. 2010b). Raw sequence data were de-multiplexed, and low quality reads were filtered using default parameters. Chimeric sequences were detected using USEARCH (Edgar 2010) and removed prior to further analysis. Sequences were assigned to operational taxonomic units (OTUs) using an open-reference picking protocol in QIIME against the Greengenes database (v. 13.8) filtered at ≥97% similarity (Caporaso et al. 2010a; DeSantis et al. 2006; Q. Wang et al. 2007).

5.2.3.1 Diversity metrics

Without filtering rare sequences, samples were rarefied to an even depth of 66,000 reads. Alpha rarefaction plots, coverage and alpha diversity metrics (Goods coverage, Chao1, Shannon, and Observed Species), and beta diversity (weighted and unweighted UniFrac distance matrices) were determined using QIIME scripts (Lozupone and Knight 2005). Variation in community distributions was visualized with Principal Coordinates Analysis (PCoA) plots based on weighted and unweighted UniFrac distances and groups were compared using ANOSIM (Vazquez-Baeza et al. 2013).

5.2.3.2 Taxonomic summaries

To simplify taxonomic summaries, OTUs present in eight or fewer of the samples were filtered out of the original unrarefied OTU table (this cut-off was chosen to conservatively eliminate taxa that are very unlikely to be core members of the microbiota or relevant to disease status). Samples were then rarefied to an even depth of 60,000 reads per sample and QIIME was used to summarize taxa at all phylogenetic levels. Taxa were analyzed in JMP (SAS, Durham, NC, USA) to the level of genus, and tested for significant differences with a Mann-Whitney *U* test. *P*-

values were adjusted using the Benjamini-Hochberg step-up method, allowing a False Discovery Rate (FDR) of 0.05.

5.2.4 Metabolomics data acquisition

Samples were analyzed on a fee-for-service basis by Metabolon (Durham, NC, USA) using multiple mass spectrometry platforms. Briefly, samples were lyophilized, deproteinized with methanol, centrifuged, and the supernatant was divided for analysis on four platforms. The four analyses were UPLC-MS/MS in both positive and negative ion modes, LC polar (HILIC), and GC-MS. The aliquot for GC-MS was dried, derivatized using bistrimethyl-silyltrifluoroacetamide, and injected on a GC fitted with a 5% diphenyl / 95% dimethyl polysiloxane fused silica column (20 m x 0.18 mm ID; 0.18 μm film thickness) with helium as carrier gas and a temperature ramp from 60° to 340°C over 17.5 min. The detector was a Thermo-Finnigan Trace DSQ fast-scanning single quadrupole MS, using electron impact ionization and scanning from 50-750 m/z. The two aliquots for UPLC-MS/MS were analyzed under acidic (water and methanol containing 0.1% formic acid) or basic (water and methanol with 6.5 mM ammonium bicarbonate) conditions, using dedicated C18 columns (Waters UPLC BEH C18-2.1 x 100 mm, 1.7 μm) for each set of conditions. The LC polar aliquot was eluted from a HILIC column (Waters UPLC BEH Amide 2.1 x 150 mm, 1.7 μm) using water and acetonitrile with 10 mM ammonium formate. The detector for all three LC-MS components was a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization source and Orbitrap mass analyzer, scanning from 80-1000 m/z. Quantification for a given signal was determined by peak area. Metabolon applied a proprietary software algorithm and library for identification of metabolites and only data for known metabolites based on their assignments were curated and reported for further analysis (Dehaven et al. 2010; Evans et al. 2009; Evans et al. 2012).

5.2.5 Analysis of metabolites

JMP was used for univariate analysis, using a Mann-Whitney *U* test to determine significance. *P*-values were adjusted using the Benjamini-Hochberg step-up method, allowing a False Discovery Rate (FDR) of 0.05. Peak area values for known metabolites were uploaded to MetaboAnalyst 3.0 (Xia Lab, McGill University, Canada) (Xia et al. 2015). The data were log transformed and Pareto scaled before statistical analyses (Xia and Wishart 2011). Multivariate analyses were performed within MetaboAnalyst and included Principal Components Analysis (PCA), Partial Least Squares-Dimensional Analysis (PLS-DA), and Random Forest Analysis (RFA). Based on ranking of features by a simple t-test, the top 50 features were used to generate a heatmap to visualize metabolomic variability across individuals within groups and between groups.

5.3 RESULTS

5.3.1 Sample population demographics

The diseased (median [range]) and healthy (median [range]) animals were not significantly different in terms of age (5.5 years [2-10] vs. 5 years [1-11], p=0.63), weight (18.2 kg [5.8-38.4] vs. 13.6 kg [5.0-30.2], p=0.43), or sex (9/15 male vs. 6/15 male, p=0.47).

5.3.2 Sequencing data

5.3.2.1 Diversity metrics

Alpha diversity was significantly decreased in feces of dogs with CE based on the metric observed_species (p=0.001). ANOSIM revealed that samples from diseased and healthy dogs were significantly different (p=0.001). Alpha diversity plots are shown in Figure 20 and a PCoA plot based on unweighted UniFrac is shown in Figure 21.

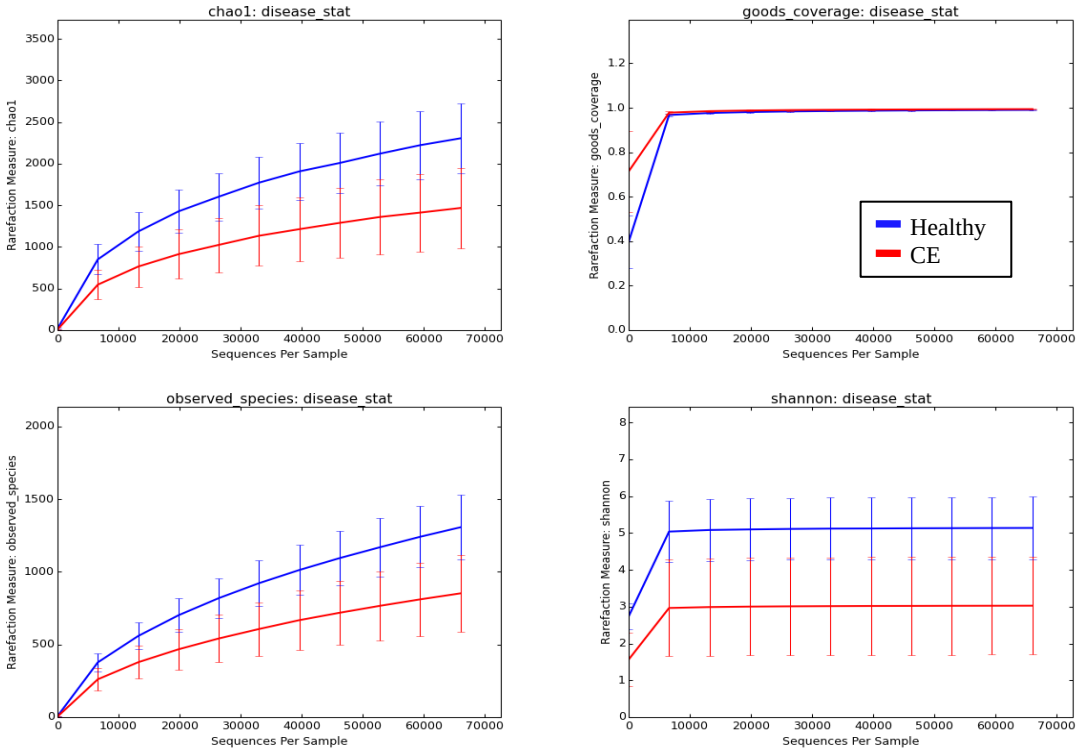


Figure 20. Alpha diversity plots comparing sequences from feces of dogs with CE to those from feces of healthy dogs.

5.3.2.2 Taxonomic summary

Summary data for taxa from phylum to genus levels are shown in Table 9. Reported values are the median percentage of total reads and the range for the samples within the group. At the level of phylum, Bacteroidetes and Fusobacteria were significantly decreased in dogs with CE (q=0.0020 and 0.0006, respectively), while the phylum Firmicutes was significantly increased (q=0.0007). While the increase in Firmicutes appears to be driven by increased Bacilli (q=0.0032), classes Clostridia and Erysipelotrichi were significantly decreased in disease (q=0.0451 and 0.0012). Although Proteobacteria were not significantly altered between groups, Betaproteobacteria significantly decreased in samples from dogs with CE while Gammaproteobacteria were significantly increased (q=0.0044 and 0.0206, respectively).

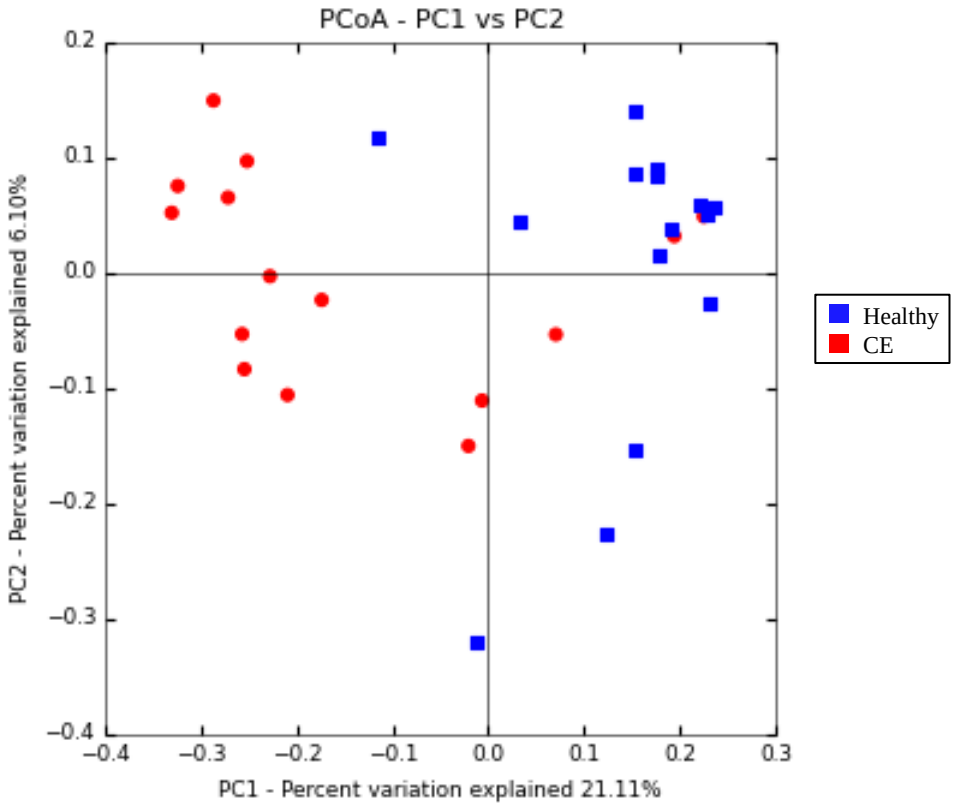


Figure 21. PCoA plot based on unweighted UniFrac distances showing slightly overlapping clustering of samples from diseased and healthy dogs.

Table 9. Taxonomic summary of bacteria sequenced from fecal DNA from dogs with chronic enteropathy and healthy control dogs. Values are the percentage of reads. *P*- and *q*-values < 0.05 are identified in boldface. "*g__*" indicates a genus of unknown identity; "f__" represents a family of unknown identity.

	Healthy		**Chronic Enteropathy**			
	Median	**Range**	**Median**	**Range**		
Taxon	**% of reads**		**% of reads**		***p*-value**	***q*-value**
Phylum Actinobacteria	1.83	[0.07-5.16]	0.42	[0.05-6.09]	**0.0465**	0.0760
Class Actinobacteria	0.02	[0.01-0.03]	0.02	[0-5.01]	0.5323	0.6294
Order Bifidobacteriales	0.02	[0.01-0.03]	0.02	[0-5.01]	0.5323	0.6294
Family Bifidobacteriaceae	0.02	[0.01-0.03]	0.02	[0-5.01]	0.5323	0.6294
Bifidobacterium	0.02	[0.01-0.03]	0.02	[0-5.01]	0.5323	0.6294
Class Coriobacteriia	1.82	[0.06-5.13]	0.06	[0.04-6.07]	**0.0016**	**0.0050**
Order Coriobacteriales	1.82	[0.06-5.13]	0.06	[0.04-6.07]	**0.0016**	**0.0050**
Family Coriobacteriaceae	1.82	[0.06-5.13]	0.06	[0.04-6.07]	**0.0016**	**0.0050**
Adlercreutzia	0.00	[0-0.07]	0.00	[0-0.01]	0.1971	0.2641
Collinsella	1.63	[0.05-4.68]	0.06	[0.03-6.07]	**0.0016**	**0.0050**
Slackia	0.01	[0-0.48]	0.01	[0-0.01]	0.4504	0.5640
Phylum Bacteroidetes	15.07	[0.18-37.52]	0.23	[0.15-28.66]	**0.0004**	**0.0020**
Class Bacteroidia	15.07	[0.18-37.52]	0.23	[0.15-28.66]	**0.0004**	**0.0020**
Order Bacteroidales	15.07	[0.18-37.52]	0.23	[0.15-28.66]	**0.0004**	**0.0020**
Family [Paraprevotellaceae]	0.29	[0.02-1.74]	0.03	[0.01-0.91]	**0.0360**	0.0661
g__	0.01	[0-0.01]	0.01	[0-0.21]	0.9662	0.9663
[Prevotella]	0.29	[0.02-1.73]	0.02	[0.01-0.9]	**0.0341**	0.0635
Family Bacteroidaceae	9.47	[0.13-37.45]	0.17	[0.1-27.71]	**0.0006**	**0.0026**
Bacteroides	9.47	[0.13-37.45]	0.17	[0.1-27.71]	**0.0006**	**0.0026**
Family Porphyromonadaceae	0.00	[0-1.82]	0.00	[0-0.03]	0.2050	0.2694
Parabacteroides	0.00	[0-1.82]	0.00	[0-0.03]	0.2050	0.2694
Family Prevotellaceae	0.12	[0.02-30.38]	0.03	[0.01-0.37]	**0.0378**	0.0676
Prevotella	0.12	[0.02-30.38]	0.03	[0.01-0.37]	**0.0378**	0.0676
Family S24-7	0.00	[0-0.03]	0.00	[0-0.01]	0.7930	0.8345
g__	0.00	[0-0.03]	0.00	[0-0.01]	0.7930	0.8345
Phylum Firmicutes	78.40	[48.48-94.89]	98.32	[55.18-99.31]	**0.0001**	**0.0007**
Class Bacilli	1.19	[0.62-80.54]	87.81	[0.73-96.96]	**0.0008**	**0.0032**
Order Bacillales	0.00	[0-0.02]	0.00	[0-0.01]	0.8327	0.8583
Family Planococcaceae	0.00	[0-0.01]	0.00	[0-0.01]	0.5900	0.6700
g__	0.00	[0-0]	0.00	[0-0]	0.3495	0.4461
Sporosarcina	0.00	[0-0]	0.00	[0-0.01]	0.7765	0.8345
Family Staphylococcaceae	0.00	[0-0.02]	0.00	[0-0.01]	0.7177	0.7819
Staphylococcus	0.00	[0-0.02]	0.00	[0-0.01]	0.7177	0.7819
Order Lactobacillales	0.63	[0.48-80.4]	87.53	[0.61-96.8]	**0.0002**	**0.0012**
f__	0.00	[0-0.01]	0.05	[0-2.27]	**0.0001**	**0.0008**
g__	0.00	[0-0.01]	0.05	[0-2.27]	**0.0001**	**0.0008**
Family Enterococcaceae	0.14	[0.1-0.41]	1.17	[0.12-89.89]	**0.0001**	**0.0006**
Enterococcus	0.02	[0.01-0.06]	0.20	[0.01-5.46]	**0.0001**	**0.0010**
Other	0.12	[0.08-0.35]	0.97	[0.11-84.43]	**0.0000**	**0.0006**
Family Lactobacillaceae	0.25	[0.18-3.63]	0.50	[0.21-90.43]	**0.0006**	**0.0026**
Lactobacillus	0.24	[0.18-3.63]	0.47	[0.21-90.43]	**0.0006**	**0.0026**
Pediococcus	0.00	[0-0.01]	0.01	[0-2.47]	**0.0050**	**0.0126**

Table 9. (Continued.)

Taxon	Healthy Median	Healthy Range	Chronic Enteropathy Median	Chronic Enteropathy Range	*p*-value	*q*-value
	% of reads		% of reads			
Family Leuconostocaceae	0.01	[0-0.02]	0.02	[0-21.37]	**0.0431**	0.0740
g__	0.01	[0-0.02]	0.02	[0-21.37]	**0.0431**	0.0740
Family Streptococcaceae	0.23	[0.16-76.34]	4.07	[0.21-82.59]	**0.0054**	**0.0130**
Streptococcus	0.23	[0.16-76.34]	4.07	[0.21-82.59]	**0.0054**	**0.0130**
Other	0.01	[0-0.02]	0.02	[0-0.3]	**0.0457**	0.0760
Other	0.01	[0-0.02]	0.02	[0-0.3]	**0.0457**	0.0760
Order Turicibacterales	0.22	[0.1-3.1]	0.16	[0.11-2.33]	0.8034	0.8345
Family Turicibacteraceae	0.22	[0.1-3.1]	0.16	[0.11-2.33]	0.8034	0.8345
Turicibacter	0.22	[0.1-3.1]	0.16	[0.11-2.33]	0.8034	0.8345
Other	0.00	[0-0]	0.00	[0-0.01]	0.0881	0.1243
Other	0.00	[0-0]	0.00	[0-0.01]	0.0881	0.1243
Other	0.00	[0-0]	0.00	[0-0.01]	0.0881	0.1243
Class Clostridia	59.15	[12.08-83.06]	6.47	[2.04-95.99]	**0.0225**	**0.0451**
Order Clostridiales	59.15	[12.08-83.06]	6.47	[2.04-95.99]	**0.0225**	**0.0451**
f__	0.90	[0.13-1.36]	0.06	[0.02-1.32]	**0.0048**	**0.0124**
g__	0.90	[0.13-1.36]	0.06	[0.02-1.32]	**0.0048**	**0.0124**
Family [Mogibacteriaceae]	0.01	[0-0.02]	0.01	[0-0.02]	0.6293	0.7028
g__	0.01	[0-0.02]	0.01	[0-0.02]	0.6293	0.7028
Family [Tissierellaceae]	0.00	[0-0.01]	0.00	[0-0.01]	0.9663	0.9663
g__	0.00	[0-0.01]	0.00	[0-0.01]	0.9663	0.9663
Family Clostridiaceae	14.72	[4.88-56.94]	2.56	[0.67-57.05]	**0.0028**	**0.0077**
g__	2.47	[0.6-41.8]	0.42	[0.13-7.3]	**0.0225**	**0.0451**
Clostridium	0.33	[0.13-11.02]	0.19	[0.06-5.32]	0.2715	0.3533
Sarcina	0.01	[0-0.14]	0.01	[0-0.02]	0.1436	0.1944
SMB53	0.27	[0.05-3.33]	0.06	[0.03-0.64]	**0.0487**	0.0787
Other	9.26	[0.78-19.91]	0.47	[0.3-48.61]	**0.0021**	**0.0061**
Family Lachnospiraceae	27.36	[6.25-39.25]	4.59	[0.48-79.59]	0.0680	0.1047
g__	1.03	[0.12-3.81]	0.07	[0.03-5.58]	**0.0019**	**0.0054**
[Ruminococcus]	2.98	[0.5-7.82]	0.23	[0.06-36.77]	0.0745	0.1134
Blautia	16.23	[2.18-23.17]	0.68	[0.24-37.81]	**0.0465**	0.0760
Coprococcus	0.03	[0-0.39]	0.00	[0-0.23]	**0.0406**	0.0716
Dorea	3.70	[0.66-9.66]	0.24	[0.09-9.82]	**0.0101**	**0.0219**
Epulopiscium	0.01	[0-5.38]	0.00	[0-0.04]	0.3353	0.4320
Roseburia	0.02	[0-0.48]	0.00	[0-0.07]	**0.0018**	**0.0054**
Other	0.30	[0.11-0.46]	0.10	[0.02-1.16]	0.1149	0.1587
Family Peptococcaceae	0.08	[0.02-6.49]	0.05	[0.03-0.1]	0.0589	0.0919
Peptococcus	0.08	[0.02-6.49]	0.05	[0.03-0.1]	0.0589	0.0919
Family Peptostreptococcaceae	0.21	[0.03-7.37]	0.04	[0.02-0.51]	**0.0089**	**0.0206**
g__	0.17	[0.03-7.35]	0.03	[0.01-0.5]	**0.0054**	**0.0130**
Peptostreptococcus	0.01	[0-0.02]	0.01	[0-0.02]	0.6438	0.7130
Other	0.01	[0-0.38]	0.00	[0-0.04]	**0.0341**	0.0635
Family Ruminococcaceae	2.82	[0.16-17.29]	0.13	[0.04-4.79]	**0.0011**	**0.0040**
g__	0.35	[0.04-1.89]	0.03	[0.01-1.7]	**0.0004**	**0.0019**
Faecalibacterium	2.21	[0.03-15.6]	0.06	[0.02-4.39]	**0.0224**	**0.0451**
Oscillospira	0.04	[0.01-0.1]	0.02	[0.01-0.08]	0.1190	0.1627
Ruminococcus	0.03	[0-0.53]	0.01	[0-0.03]	**0.0018**	**0.0054**
Family Veillonellaceae	1.23	[0.06-21.4]	0.06	[0.04-2.97]	**0.0003**	**0.0019**
Megamonas	0.92	[0.05-21.39]	0.05	[0.03-2.96]	**0.0003**	**0.0017**
Phascolarctobacterium	0.07	[0.01-0.76]	0.01	[0-0.35]	0.1002	0.1399

Table 9. (Continued.)

Taxon	Healthy Median	Healthy Range	Chronic Enteropathy Median	Chronic Enteropathy Range	*p*-value	*q*-value
	% of reads		% of reads			
Other	0.09	[0.02-0.59]	0.06	[0.01-0.1]	**0.0324**	0.0622
Other	0.09	[0.02-0.59]	0.06	[0.01-0.1]	**0.0324**	0.0622
Class Erysipelotrichi	6.81	[0.66-23.16]	0.32	[0.17-9.5]	**0.0002**	**0.0012**
Order Erysipelotrichales	6.81	[0.66-23.16]	0.32	[0.17-9.5]	**0.0002**	**0.0012**
Family Erysipelotrichaceae	6.81	[0.66-23.16]	0.32	[0.17-9.5]	**0.0002**	**0.0012**
g__	1.24	[0.05-6.47]	0.07	[0.04-1.75]	**0.0010**	**0.0040**
[Eubacterium]	1.18	[0.18-10.91]	0.10	[0.05-6.97]	**0.0003**	**0.0017**
Allobaculum	0.78	[0.05-6.2]	0.07	[0.04-2.31]	0.0590	0.0919
Bulleidia	0.00	[0-0]	0.00	[0-0]	0.3554	0.4492
Catenibacterium	1.35	[0.02-17.93]	0.04	[0.02-2.54]	**0.0212**	**0.0451**
Clostridium	0.00	[0-0.01]	0.00	[0-0.01]	**0.0023**	**0.0065**
Coprobacillus	0.02	[0-0.72]	0.00	[0-0.02]	**0.0325**	0.0622
Erysipelothrix	0.01	[0-0.02]	0.01	[0-0.02]	0.4761	0.5907
Phylum Fusobacteria	4.45	[1.29-16.9]	0.28	[0.21-9.85]	**0.0001**	**0.0006**
Class Fusobacteriia	4.45	[1.29-16.9]	0.28	[0.21-9.85]	**0.0001**	**0.0006**
Order Fusobacteriales	4.45	[1.29-16.9]	0.28	[0.21-9.85]	**0.0001**	**0.0006**
Family Fusobacteriaceae	4.45	[1.29-16.9]	0.28	[0.21-9.85]	**0.0001**	**0.0006**
Fusobacterium	4.41	[1.28-16.84]	0.28	[0.21-9.78]	**0.0001**	**0.0006**
Other	0.04	[0.01-0.09]	0.00	[0-0.06]	**0.0000**	**0.0006**
Phylum Proteobacteria	0.21	[0.01-1.33]	0.20	[0.01-9.19]	0.9174	0.9384
Class Betaproteobacteria	0.16	[0-0.59]	0.01	[0-0.19]	**0.0013**	**0.0044**
Order Burkholderiales	0.16	[0-0.59]	0.01	[0-0.19]	**0.0013**	**0.0044**
Family Alcaligenaceae	0.16	[0-0.59]	0.01	[0-0.19]	**0.0013**	**0.0044**
Sutterella	0.16	[0-0.59]	0.01	[0-0.19]	**0.0013**	**0.0044**
Class Epsilonproteobacteria	0.00	[0-0.04]	0.00	[0-0.01]	0.0792	0.1154
Order Campylobacterales	0.00	[0-0.04]	0.00	[0-0.01]	0.0792	0.1154
Family Helicobacteraceae	0.00	[0-0.04]	0.00	[0-0.01]	0.0792	0.1154
Helicobacter	0.00	[0-0.04]	0.00	[0-0.01]	0.0792	0.1154
Class Gammaproteobacteria	0.01	[0-0.72]	0.14	[0.01-9.18]	**0.0092**	**0.0206**
Order Enterobacteriales	0.01	[0-0.72]	0.14	[0.01-9.18]	**0.0092**	**0.0206**
Family Enterobacteriaceae	0.01	[0-0.72]	0.14	[0.01-9.18]	**0.0092**	**0.0206**
g__	0.01	[0-0.72]	0.13	[0.01-9.17]	**0.0098**	**0.0215**
Other	0.00	[0-0]	0.00	[0-0.02]	**0.0033**	**0.0089**
Phylum Tenericutes	0.00	[0-0.01]	0.00	[0-1.2]	0.5495	0.6294
Class Mollicutes	0.00	[0-0.01]	0.00	[0-1.2]	0.5495	0.6294
Order Anaeroplasmatales	0.00	[0-0.01]	0.00	[0-1.2]	0.5495	0.6294
Family Anaeroplasmataceae	0.00	[0-0.01]	0.00	[0-1.2]	0.5495	0.6294
Anaeroplasma	0.00	[0-0.01]	0.00	[0-1.2]	0.5495	0.6294

5.3.3 Metabolomics

5.3.3.1 Univariate analysis

A total of 787 named biochemical compounds were identified. Of these, 269 had $p<0.05$, and 145 were significantly altered after adjusting for multiple comparisons ($q<0.05$). The median normalized peak areas and ranges for named metabolites are shown in the Appendix, Table A-2, along with their corresponding *p*- and *q*-values.

5.3.3.2 Multivariate analysis

The PCA plot comparing the unsupervised grouping of samples is shown in Figure 22 along with the PLS-DA plot, which is a supervised method, showing much more distinct separation. To visualize the distribution of specific metabolites responsible for the characterization of the samples, a heatmap made up of the top 50 ranked features (by *t*-test) is shown in Figure 23.

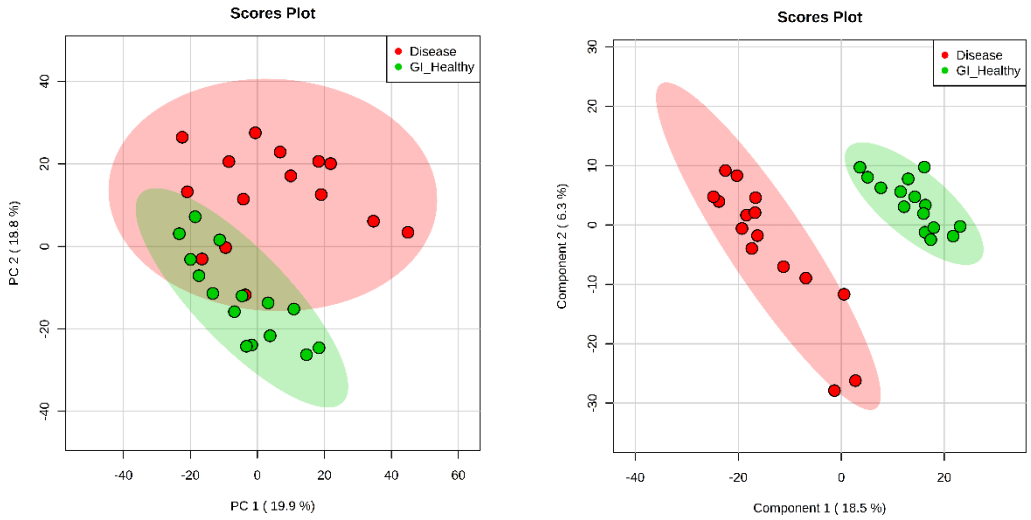


Figure 22. Clustering of samples using PCA and PLS-DA plots. The PCA plot (left) employs an unsupervised method and groups overlap slightly, while clear separation is evident in the PLS-DA plot (right) using a supervised method.

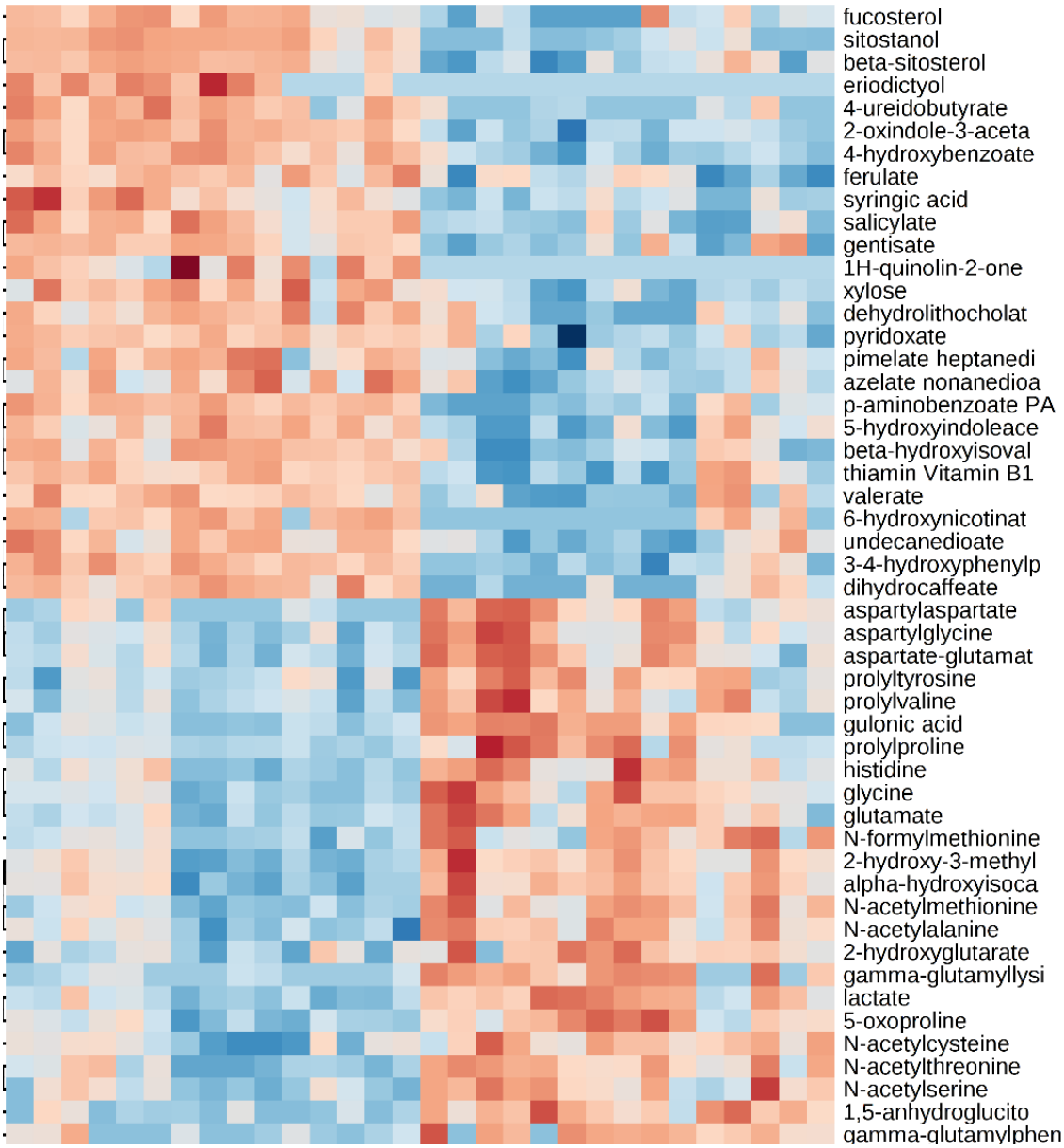


Figure 23. Heatmap using the top 50 features as ranked by a t-test. Each column corresponds to an individual sample, with the left half corresponding to 15 healthy control dogs and the right half corresponding to 15 dogs with chronic enteropathy. Metabolites are displayed in rows, where red shades indicates a relative increase in abundance and blue shades indicate a relative decrease in abundance.

As described previously, identifying metabolites as significant using more than one statistical analysis method adds support to the metabolite alteration being genuine. The top 50 metabolites ranked each by PLS-DA VIP scores and RFA mean decrease accuracy are compared in Table 10.

Table 10. The 24 compounds that were ranked among the top 50 metabolites by both RFA and PLS-DA**.**

Compound	**RFA rank**	**PLS-DA rank**
3-4-hydroxyphenylpropionate	1	9
2-oxindole-3-acetate	2	1
p-aminobenzoate PABA	3	4
4-hydroxybenzoate	4	6
sitostanol	5	13
1,5-anhydroglucitol 1,5-AG	6	20
xylose	8	22
thiamin Vitamin B1	9	10
N-acetylmethionine	10	25
lactate	11	3
5-2-Hydroxyethyl-4-methylthiazole	14	34
N-acetylalanine	20	44
isoferulate	21	38
gamma-glutamyllysine	24	7
gamma-glutamylphenylalanine	25	23
tyramine	26	49
aspartylaspartate	27	8
syringic acid	31	15
gentisate	32	41
2-hydroxy-3-methylvalerate	33	17
glycine	36	47
aspartylglycine	45	26
alpha-hydroxyisocaproate	46	16
eriodictyol	47	18

5.4 DISCUSSION

5.4.1 Sequencing

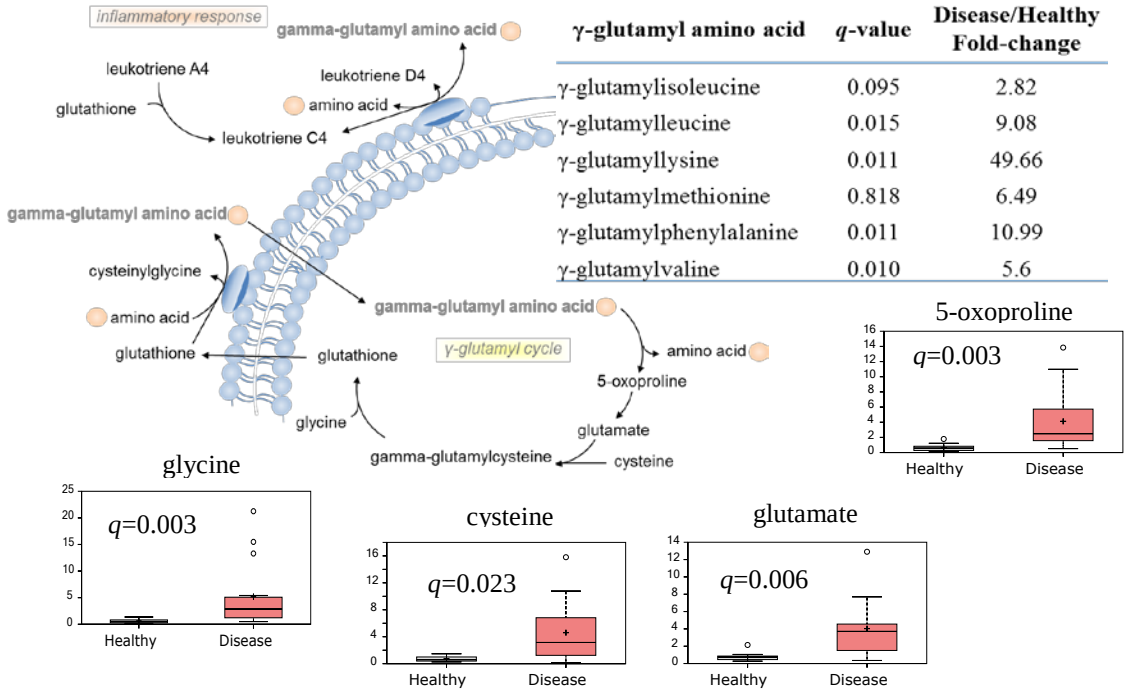
The results of 16S rRNA sequencing reported in this study are not vastly dissimilar from previously reported results comparing samples from healthy dogs to those from dogs with chronic enteropathy. One somewhat surprising finding was the increased percentage of sequences corresponding to the phylum Firmicutes in samples from diseased dogs. However, this was largely driven by profoundly increased groups within the order Lactobacillales, while the groups Bacteroidia, Clostridia, and Erysipelotrichi were decreased as has been previously reported in both human IBD and canine CE (Minamoto et al. 2015; Honneffer et al. 2014; AlShawaqfeh et al. 2016; Dandrieux 2016). This inconsistency regarding Lactobacillales has previously been reported for humans with IBD (W. Wang et al. 2014). In the present study, there were subjectively some inconsistencies between the owner and referring veterinarian questionnaires regarding the reporting of medications, so one possibility is that this particular cohort of dogs with CE had been treated more intensively with antibiotics, probiotics, or other medications that might influence the microbiota. Metronidazole in particular has been shown to greatly increase fecal lactate via increased lactic acid bacteria (Suchodolski et al. 2016). Regardless, the results of the 16S rRNA sequencing in the present study are not inconsistent with previously reported studies in dogs with CE.

5.4.2 Metabolomics

Alterations in the fecal microbiota have repeatedly been identified in both human patients with IBD and dogs with CE. However, the mechanism by which the bacteria play a role in disease has not been elucidated. Considering the microbiota as a metabolically active organ, a shift in the collective function of the host and microbiota may manifest as altered biochemical composition

within the lumen of the GI tract and this may play a role in the pathogenesis of disease. Unfortunately, this is simultaneously one of the weaknesses of metabolomics: while the biochemical composition can be determined without concern for the origin of the metabolite (host, microbiota, or both), it also means that interpretation of potential causes for an altered composition is greatly complicated. Some studies take care to determine which metabolites are thought to be of a specific origin, as done by Sridharan et al. (2014) with a focus on aromatic amino acid metabolites. Therefore, an attempt to summarize a biological interpretation of the entire untargeted metabolomics data set would quickly become an intense tangle of trans-kingdom biochemical webs. Instead, some metabolites can be grouped together based on a known biochemical or physiological relationship (e.g., redox homeostasis) for proposed interpretation. It should be noted, however, that even these proposed interpretations require much more rigorous experimentation before considering them as anything more than fanciful ideas.

Among major metabolic pathways, some metabolites associated with GIT redox homeostasis were altered, including significantly increased concentrations of precursors of glutathione: 5-oxoproline (q=0.004), cysteine (q=0.024), glycine (q=0.003), and γ-glutamyl amino acid derivatives of lysine, phenylalanine, valine, and leucine (all $q \leq 0.015$). The γ-glutamyl amino acids are generated by gamma-glutamyl transpeptidase (GGT), which is located on the outer surface of the cell plasma membrane and has extracellular activity (Zhang et al. 2005). The host peroxisome is implicated in redox metabolism, but also plays a significant role in β-oxidation of fatty acids (Deb and Nagotu 2017). A schematic of the redox cycle and the distribution of samples for each of these compounds are shown in Figure 24.



γ-glutamyl amino acid	*q*-value	Disease/Healthy Fold-change
γ-glutamylisoleucine	0.095	2.82
γ-glutamylleucine	0.015	9.08
γ-glutamyllysine	0.011	49.66
γ-glutamylmethionine	0.818	6.49
γ-glutamylphenylalanine	0.011	10.99
γ-glutamylvaline	0.010	5.6

Figure 24. Redox homeostasis and metabolite changes in dogs with CE compared to healthy control dogs.

Primary bile acids were generally increased in dogs with disease (cholate, q=0.013; chenodeoxycholate, q=0.055), while secondary bile acids were generally decreased in dogs with chronic enteropathy (deoxycholate, q=0.045; lithocholate, q=0.059), suggesting impaired bacterial conversion from primary to secondary bile acids. Recent and ongoing work in our lab has identified a very strong relationship between *Clostridium hiranonis* and bile acid profiles (unpublished work). Though the data in the present study were not shown since species-level assignment of taxa is somewhat unreliable, *C. hiranonis* was significantly decreased in the dogs with CE (Mann-Whitney *U* test resulted in p=0.0095, uncorrected for multiple comparisons). Also of interest is that there were three dogs with CE that had normal *C. hiranonis* sequence percentages, and two of those three dogs also fell well within the 95% confidence region for

healthy dogs on the global metabolite profiles represented by PCA plots (see Figure 8). Although far from being conclusive evidence, it is interesting that out of a 787-feature metabolome composition and over 200-taxa microbiota composition, certain key features may account for classification and warrant further targeted inspection, particularly to determine if perhaps the cause of chronic GI signs in these individuals is of an alternative etiology.

Other metabolites exhibiting significant alterations included those within the aromatic amino acid biosynthesis pathway. The tryptophan metabolites indoleacetate, 5-hydroxyindoleacetate, and indolepropionate were significantly decreased in dogs with disease (q=0.008, 0.008, and 0.030, respectively), though tryptophan and indolelactate trended up in diseased animals (q=0.156 and 0.137, respectively). 2-oxindole-3-acetate, an oxidative degradation product of indoleacetate, was 100-fold decreased in diseased animals (q=0.001) and was very highly ranked for discriminating power out of all detected metabolites. Among the data for gene expression in duodenal biopsies of dogs with IBD (Wilke et al. 2012), the gene SLC16A10 exhibited a 4-fold increase in expression in dogs with IBD. This gene encodes a sodium-independent aromatic amino acid transporter. Although the gene expression data is host-specific and the data are based on duodenal tissues, while the metabolomics data corresponds to feces, it is possible that the increased transporter expression is depriving the luminal contents of those aromatic amino acids. Conversely, the gene expression data also showed a 3.5-fold decrease in SLC1A1, the excitatory amino-acid transporter 3 (EAAT3). EAAT3 is a major route of neuronal cysteine uptake (Guitart et al. 2015), also potentially related to the previously mentioned redox homeostasis and potentially a partial cause for increased fecal cysteine among dogs with CE. Additionally, the ability of the microbiota to metabolize amino acids has also been described (Mead 1971; Smith and Macfarlane 1996; Sridharan et al. 2014). Regardless, the pattern of certain amino acids being increased while others were decreased does suggest that the cause is not just a

simple change in absorptive ability for the host, and further supports the idea that some metabolites are bioactive and their presence may influence other biochemical relationships.

Also evident in the data and particularly relevant to our ongoing research are potential alterations in lipid metabolism in dogs with CE, which have been shown to some extent in human IBD patients (Shores et al. 2011). Several classes of lipids were altered in disease, to varying levels of significance. Saturated LCFAs palmitic and stearic acids were increased in disease (q=0.156 and 0.022); monounsaturated VLCFAs erucic and eicosenoic (also known as gondoic) acids also were increased. Interestingly, among PUFAs there were significant increases of both *n*-3 and *n*-6 fatty acids: docosatrienoate (22:3 *n*-3, q=0.020), arachidonate (20:4 *n*-6, q=0.045), and docosadienoate (22:2 *n*-6, q=0.060). It was mentioned previously that ω-oxidation of fatty acids results in conversion of the terminal methyl group on a fatty acid to a second carboxylic acid group. Interestingly, saturated dicarboxylic fatty acids from seven to eleven carbons in length were decreased among animals with disease: pimelate, suberate, azelate, sebacate, and undecanedioate (q=0.007, 0.049, 0.007, 0.020, and 0.005, respectively). Of the corresponding saturated MCFAs, only C8 (caprylic acid) and C10 (capric acid) were reported in the data, and were not significantly different between healthy and diseased animals. This may indicate alterations in ω-oxidation of fatty acids in diseased animals, but the MCFAs also could have been diverted to other products without necessarily an underlying defect in ω-oxidation pathways. Interestingly, the primary degradation of fatty acids occurs in the peroxisome by β-oxidation, and the peroxisome is also credited with a role in redox homeostasis along with many other functions (Deb and Nagotu 2017). Other altered lipids include some sterols. Interestingly, cholesterol trended to higher concentrations in feces of dogs with CE (q=0.128) while coprostanol (the hydrogenated form of cholesterol) trended to lower concentrations (q=0.190). A correlation between cholesterol-to-coprostanol conversion and GI health has been shown in people (Antharam et al. 2016; Olejníková

et al. 2017), but the dog supposedly does not host the microbiota capable of biohydrogenating cholesterol, so it is unclear whether this trend is suggestive of a similar situation in the dog, or if coprophagia (ingestion of coprostanol from eating other animal's feces) could be a confounding factor. Additionally, several phytosterols were decreased: β-sitosterol (q=0.003), campesterol (q=0.067), fucosterol (q=0.006), and sitostanol (q=0.001). Because these sterols are associated with plants, an association with diet should be considered, but it is also conceivable that the net effects of intraluminal sterol homeostasis are affected by disease, and further exploration of these metabolites is warranted.

5.5 CONCLUSIONS

This study demonstrates that feces are a rich sample matrix for biomolecules, representing both the host and microbial metabolite profiles. Several metabolites were identified that may be investigated as future biomarkers and may help elucidate the etiopathogenesis of canine chronic enteropathy. Specifically of interest are compounds associated with lipid metabolism: bile acids, sterols, and fatty acids.

6. DEVELOPMENT AND VALIDATION OF A GAS CHROMATOGRAPHY-MASS SPECTROMETRY ASSAY FOR THE QUANTIFICATION OF STEROL AND FATTY ACID CONCENTRATIONS IN CANINE FECES

OVERVIEW

Cholesterol is the most abundant sterol in mammalian tissues, playing a critical role in cell membrane function and serving as a precursor molecule for hormones, vitamins, and bile acids. Structurally similar to cholesterol, phytosterols have been associated with anti-inflammatory effects in rodent models of colitis. Previous studies have shown altered fecal sterol concentrations in dogs with chronic enteropathy. Fatty acids are an important source of energy, and along with sterols, they are also critical for intestinal epithelial cell membrane structure and function. The aim of this study was to analytically validate a gas chromatography-mass spectrometry (GC-MS) assay for the measurement of several sterols (i.e., cholesterol, coprostanol, cholestanol, β-sitosterol, stigmasterol, campesterol, sitostanol, and fucosterol) and fatty acids (i.e., palmitic, oleic, linoleic, α-linolenic, stearic, gondoic, and erucic acids) in canine fecal samples.

A fecal sample was collected from eight dogs, lyophilized, and used for assessment of intra- and inter-assay variability (%CV). Sample and standard preparation were adapted from published methods, using deuterated analogs of cholesterol, sitostanol, and stearic acid for internal standards. Validation parameters also included lower and upper limits of quantitation (LLOQ and ULOQ, respectively). Quality control procedure for each sample run consisted of blanks, continuing calibration verification of standards, and a laboratory control sample of pooled feces.

Intra- and inter-assay variability (median %CV) were 4% and 6% for cholesterol, 5% and 10% for coprostanol, 4% and 9% for cholestanol, 4% and 5% for β-sitosterol, 4% and 7% for stigmasterol, 4% and 6% for campesterol, 4% and 11% for sitostanol, 6% and 10% for fucosterol,

4% and 7% for palmitic acid, 4% and 8% for oleic acid, 8% and 7% for linoleic acid, 14% and 12% for α-linolenic acid, 4% and 6% for stearic acid, 5% and 11% for gondoic acid, and 10% and 18% for erucic acid. Limits of quantitation for each compound in μg/mL (LLOQ-ULOQ) were as follows: palmitic acid (7.8-2,000), linoleic acid (15.6-2,000), α-linolenic acid (3.1-200), oleic acid (7.8-2,000), stearic acid (3.9-2,000), gondoic acid (0.39-200), erucic acid (0.78-200), cholesterol (3.9-2,000), campesterol (6.3-400), stigmasterol (0.78-100), fucosterol (1.6-200), β-sitosterol (12.5-800), coprostanol (0.78-200), cholestanol (3.1-200), and sitostanol (3.1-200).

In conclusion, the GC-MS method validated here is precise, reproducible, and sensitive for the quantification of several sterols and fatty acids in canine fecal samples.

6.1 INTRODUCTION

Sterols and fatty acids are two subclasses of the biochemicals known as lipids, having the defining feature of insolubility in water. Given the aqueous nature of most organisms, there is an inherent conflict in the dependence on lipids – yet the hydrophobic nature of lipids is also what permits formation of barriers, such as cell membranes. Cholesterol, the most abundant sterol in mammalian tissues, is an important precursor for bile acids, vitamins, and hormones. It also plays a role in formation of lipid rafts, which are part of cell signaling regulation (Lingwood and Simons 2010). Fatty acids are critical for cell structure as well, comprising much of the phospholipid bilayer, and also serve as a substrate for generation of energy in mitochondria. Based on an untargeted metabolomics study comparing feces from dogs with chronic enteropathy to those of healthy control dogs (described in Section 5), sterols and fatty acids were identified as potential biomarkers for disease. However, untargeted metabolomics is only semi-quantitative, allowing comparison of abundances of a given metabolite between samples analyzed together, but not

allowing determination of actual concentrations so individual samples could be compared to a reference interval for healthy dogs.

Fecal matter is a complex matrix for analysis, making assay development a balance between user practicality and chemical perfection: multiple steps of homogenization and cleanup are likely to improve reproducibility of the assay, but also complicate and lengthen the preparation. Liquid extraction methods typically produce large amounts of waste containing organic solvents and may not be consistently quantitative. In contrast, solid phase extraction requires selection of the appropriate column and elution conditions, and we had concerns that the variability of the fecal matrix would introduce confounding variability or require initial cleanup steps prior to extraction. For the assay to have future utility to clinicians, it would need to be affordable and have a short turnaround time, so we sought a simple sample preparation method. The most appealing method we found as a starting point was designed for gas chromatography with a flame ionization detector (GC-FID), but simultaneously quantified fatty acids, sterols, and bile acids from feces (Batta et al. 2002; Batta et al. 1999). The general approach was to convert acid groups to their butyl esters, and subsequently derivatize remaining hydroxyl groups to trimethylsilyl ethers. The authors suggested that these steps improved volatility of the compounds and enhanced chromatographic characteristics of the peaks (resolution and shape) relative to the underivatized compounds. Here we present the adaptation, development, and validation of a quantitative assay using gas chromatography-mass spectrometry (GC-MS) to analyze canine fecal samples for a variety of sterols and fatty acids.

6.2 MATERIALS AND METHODS

6.2.1 Instrumentation

A model 6890N gas chromatograph (GC; Agilent, Santa Clara, CA, USA) coupled with a model 5975 mass selective detector (MSD) with inert electron impact (EI) ionization and a 7683B autosampler were used for all analyses. GC operating conditions were as follows: capillary column DB-1MS UI (Agilent, 30 m x 0.25 mm I.D. and 0.25 μm film thickness) and a split liner (taper, low pressure drop, with glass wool). Helium was used as the carrier gas at 1 mL/min; a 1 μL volume of sample was injected in a 20:1 split; inlet temperature at 250°C. Oven temperature was initially held at 150°C for 1 minute, ramped to 276°C at 21°C/minute, and held for 15 minutes with a post-run time of 3 minutes at 325°C. The relationship between retention time of d_4-cholestane and column pressure was calibrated and pressure was adjusted as needed to maintain consistent retention time (RTLock). The detector was tuned daily with perfluorotributylamine (PFTBA) using the default AutoTune algorithm, and MSD parameters included transfer line temperature 230°C and solvent delay of 3 minutes. Commercially-sourced compounds were characterized in Scan mode (50-550 m/z) to confirm purity and identify characteristic ion peaks. The MS was run in SIM mode (selected ion monitoring) for quantitative analysis, using ion fragments for quantitation and verification as described in Table 11.

Table 11. Quantified compounds and characteristics of the ion fragments used, ordered by increasing retention time. For fatty acids, the number of carbons and units of unsaturation are also specified.

Compound	Retention Time (min)	Quantitation ion (nominal m/z)	Qualifying ions (nominal m/z [% intensity])
palmitic acid (16:0)	6.9	257	312[20], 239[51]
linoleic acid (18:2, n-6)	7.6	263	262[49], 336[32]
alpha-linolenic acid (18:3, n-3)	7.6	261	79[1100], 334[59]
oleic acid (18:1, n-9)	7.7	264	265[120], 22[41]
cis-vaccenic acid (18:1, n-7)	7.7	265	264[78], 338[11], 79[16]
d_4-stearic acid (ISTD)	7.8	289	344[33], 271[49]
stearic acid (18:0)	7.8	285	340[33]
arachidonic acid (20:4, n-6)	8.4	292	150 [N/A]
gondoic acid (20:1, n-9)	8.7	293	292[91], 250[23], 366[12]
erucic acid (22:1, n-9)	10.2	321	320[94], 236[21], 394[10]
behenic acid (22:0)	10.4	341	396[65], 323[42]
d_4-cholestane (RTLock)	11.4	361	221[239], 376[75]
nervonic acid (24:1, n-9)	12.3	348	349[97]
coprostanol	14.2	370	355[41], 257[16]
d_6-cholesterol (ISTD)	15.7	464	333[219], 359[169], 374[241]
cholesterol	15.8	368	353[52], 329[130]
cholestanol	16.0	445	460[69], 355[59]
brassicasterol	16.6	TIC	470[31], 380[34], 341[21]
lathosterol	16.9	458	443[24], 213[31]
campesterol	18.0	343	382[85], 367[41]
stigmasterol	18.8	484	394[99], 469[18]
fucosterol	20.3	386	296[61]
β-sitosterol	20.3	357	396[91], 381[40]
d_7-sitostanol (ISTD)	20.5	480	390[70], 405[38]
sitostanol	20.7	473	488[87], 383[66]

6.2.2 Chemicals and reagents

All reagents and solvents were of analytical-reagent grade or maximum available purity. Fatty acids and sterols were obtained from Sigma-Aldrich (St. Louis, MO, USA), ChromaDex (Irvine, CA, USA), and Steraloids (Newport, RI, USA). Deuterated internal standards were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Sylon HTP (a 3:1:9 mixture of hexamethyldisilazane, trimethylchlorosilane, and anhydrous pyridine packaged in glass ampules) was purchased from Sigma-Aldrich. Concentrated hydrochloric acid (HCl, 37%) and

solvents of HPLC-grade purity were purchased from Sigma-Aldrich or VWR (Radnor, PA, USA). No further purification was performed.

6.2.3 Fecal sample collection

Naturally passed fecal samples were collected and frozen at -80°C. Samples for assay validation were surplus from unrelated studies. A 0.5-1.0 g aliquot of feces was frozen overnight at -80°C, then lyophilized overnight, and finally homogenized with a flexible disposable plastic spatula before weighing for analysis.

6.2.4 Stock and standard working solutions

Stock solutions of 2 mg/mL in chloroform were prepared of each target compound and stored at -20°C. Working solutions for calibration curves were prepared as a series of two-fold dilutions with physiologically-relevant high-concentration standards (see limits of quantitation in 4.3.1). Internal standards were prepared at 2 mg/mL in chloroform (d_6-cholesterol, d_7-sitostanol, and d_4-stearic acid) or hexane (d_4-cholestane) and stored at -20°C.

6.2.5 Preparation of standard samples

6.2.5.1 Sterol standard samples

Appropriate volumes of 2 mg/mL stock solutions of the sterols were combined (in groups) and dried under N_2 gas flow, then reconstituted with 1 mL chloroform. A series of two-fold dilutions was prepared in chloroform and 100 µL of each transferred to glass tubes. A master-mix of the relevant IS (d_6-cholesterol and/or d_7-sitostanol as appropriate) was prepared such that a 20 µL volume would result in the final 100 µg/mL IS concentration. Standard samples were then dried with heat and N_2 flow before adding 100 µL Sylon-HTP. Tubes were capped and incubated at 65°C for 30 minutes. Sterol standard samples were again dried and reconstituted in 100 µL hexane for injection onto the instrument. It was found that some of the sterols were subject to degradation

if heated with HCl (in the absence of a fecal matrix), so the calibration curves for sterols were prepared with omission of the butyl esterification step.

6.2.5.2 Fatty acid standard samples

Appropriate volumes of 2 mg/mL stock solutions of the fatty acids were combined (in groups) and dried under N_2 gas flow, then reconstituted with chloroform. For calibration, this mixed stock was dried and resuspended in an equivalent volume of butanol. A series of two-fold dilutions was prepared in butanol and 100 μL of each transferred to glass tubes. A master-mix of butanol, d4-stearic acid IS, and HCl was prepared such that each tube received 5 μL IS, 20 μL HCl, and 95 μL butanol. Standard samples were capped and incubated at 65°C for four hours. Samples were subsequently dried by heating under N_2 gas flow, then reconstituted in 100 μL hexane. Since the fatty acids analyzed were inert to trimethylsilyl derivatization after butyl esterification, the addition of Sylon-HTP was omitted when preparing the standard samples for fatty acid calibration curves.

6.2.6 Quality control (QC) samples

QC consisted of a set of five samples: prep blank (PB), zero blank (ZB), sterol continuing calibration verification (S-CCV), fatty acid continuing calibration verification (F-CCV), and laboratory control sample (LCS). The PB contained only butanol and hydrochloric acid. The ZB was equivalent to the PB with addition of IS. Both blanks were used to assess contamination throughout the preparation and analysis. The S-CCV and F-CCV consisted of mixes of stock solutions of sterols and fatty acids, respectively, of known concentrations within the limits of quantitation. The LCS consisted of lyophilized feces that were pooled, ground with a mortar and pestle, and stored frozen. With the exception of additions of HCl to the S-CCV and master mix to the PB, the QC samples were prepared alongside the unknown fecal samples as described in Section 2.7.

6.2.7 Sample preparation

A sample batch consisted of the 5 QC samples and up to 22 unknown fecal samples. Aliquots of 10-15 mg lyophilized feces were precisely weighed into disposable glass centrifuge tubes. A master mix was prepared in sufficient quantity for all samples to receive 160 µL butanol and 10 µL each d_7-sitostanol, d_6-cholesterol, d_4-stearic acid, and d_4-cholestane. The master mix was thoroughly mixed, then added to each tube except the PB, which instead received 200 µL butanol. All tubes except the S-CCV then received 20 µL concentrated HCl. Tubes were capped and vortexed at least 30 seconds before incubating at 65°C for 4 hours to convert carboxylic acid groups to butyl esters. Samples were briefly vortexed to mix, then dried with heat under N_2 until visibly dry. 200 µL of Sylon-HTP was added to each dried sample, which were then capped, vortexed, and incubated at 65°C for 30 minutes to convert sterol alcohol groups to their trimethylsilyl (TMS) derivatives. Samples were again vortexed briefly before drying under heat and N_2 flow. 200 µL hexane was then added to each tube and mixed. Tubes were then centrifuged at 3,200 rcf and 5°C for 10 minutes. 80-100 µL of supernatant was transferred to a glass GC vial insert for injection and analysis.

6.2.8 Data analysis

Total ion current (TIC) chromatograms from each batch were overlaid to verify consistent retention time of the RTLock compound, d_4-cholestane, and a consistent baseline. The software (ChemStation, Agilent) was used to automatically integrate all peaks and calculate the concentrations of analytes (µg/mL) in the injected hexane solution. Validity of peak assignments was assessed by comparing expected retention times and relative ion intensities, and manual reintegration of selected peaks was performed when faulty automatic assignment was suspected (e.g., two compounds assigned to the same retention time). These data were exported and the original mass of lyophilized feces for each sample was used to calculate concentrations in µg per

mg of lyophilized feces. The TIC of the PB was assessed for contamination subjectively, while the ZB was assessed for contamination exceeding the lower limit of quantitation. The validity of the calibration curves and sample preparation was assessed by confirming CCV concentrations were within 20% of the expected values, and the LCS was within 2 standard deviations of the mean values (based on a minimum of 20 previous analyses of the LCS).

6.3 RESULTS

6.3.1 Limits of detection and quantitation

The limit of detection (LOD) was defined as the calculated concentration resulting from the response ratio of the mean signal of the zero blank (n=20) plus 10 times the standard deviation of the blank. This calculated method based on noise was used since the number of analytes in the panel and range of concentrations would make experimental determination using a low-concentration sample for all analytes impractical. The lower and upper limits of quantitation (LLOQ and ULOQ, respectively) were defined as the lowest and highest concentrations of each target analyte that could be measured with acceptable accuracy (within 20% of actual concentration). These values are reported in Table 12.

6.3.2 Dilutional parallelism

The lyophilized feces were diluted with alumina powder (Al_2O_3, Agilent) in series by weight (a portion of the 1:1 mixture of feces:Al_2O_3 was combined with an equal weight of pure Al_2O_3 to prepare the 1:3 mixture). A 10-15 mg aliquot of each of the samples from five animals and their dilution series were prepared as described in Section 2.7. The results in μg/mg lyophilized sample and the observed-to-expected ratios are reported in Table 13.

6.3.3 Spiking recovery

Spiking recovery was determined by preparing combinations of pairs of fecal samples from four different animals, with the expected results calculated as a weighted average of the mass of each fecal sample used. The results in μg/mg lyophilized sample and the observed-to-expected ratios are reported in Table 14 and Table 15.

6.3.4 Precision

Precision was assessed by intra-assay variability and reproducibility was assessed by evaluation inter-assay variability as determined by the coefficient of variation (%CV) of six replicates of each of six samples prepared and analyzed on the same (intra-assay) or different (inter-assay) preparation batches and instrument run dates. Values that were outside the limits of quantitation were used without adjustment. The median and range of the samples that were tested, and the resultant median and range of the %CV, are reported in Table 16.

Table 12. Limits of detection and quantitation.LOD: Limit of detection, calculated from the mean signal of the blank +10 SD. LLOQ: lower limit of quantitation. ULOQ: upper limit of quantitation. $LOQ_{12.5}$: the equivalent LOQ for feces if 12.5 mg were used.

Compound	LOD µg/mL	LLOQ µg/mL	ULOQ µg/mL	$LLOQ_{12.5}$ µg/mg	$ULOQ_{12.5}$ µg/mg
palmitic acid	4.90	7.8125	2000	0.125	32
linoleic acid	7.85	15.625	2000	0.25	32
α-linolenic acid	1.51	3.125	200	0.05	3.2
oleic acid	4.46	7.8125	2000	0.125	32
cis-vaccenic acid	4.95	6.25	400	0.1	6.4
stearic acid	4.67	7.8125	2000	0.1250	32
arachidonic acid	13.04	25.0	800	0.4	12.8
gondoic acid	0.13	0.3906	200	0.00625	3.2
erucic acid	0.05	0.78125	200	0.0125	3.2
behenic acid	4.94	6.25	800	0.1	12.8
nervonic acid	4.48	12.5	800	0.2	12.8
cholesterol	1.19	3.906	2000	0.0625	32
brassicasterol	0.11	0.3906	100	0.00625	1.6
lathosterol	0.30	0.78125	100	0.0125	1.6
campesterol	2.39	6.25	400	0.1	6.4
stigmasterol	0.39	0.78125	100	0.0125	1.6
fucosterol	0.70	1.5625	200	0.025	3.2
β-sitosterol	7.14	12.5	800	0.2	12.8
coprostanol	1.38	1.5625	200	0.0250	3.2
cholestanol	1.05	3.125	200	0.05	3.2
sitostanol	0.36	3.125	200	0.05	3.2

Table 13. Dilutional parallelism. Observed and expected values are expressed in μg/mg lyophilized sample weight.

		palmitic acid			linoleic acid			α-linolenic acid			oleic acid			*cis*-vaccenic acid		
Animal	**Feces:Al_2O_3**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**
I	1:0	30.691			5.908			0.263			11.868			8.621		
	1:1	15.534	15.345	101%	2.959	2.954	100%	0.119	0.131	91%	5.677	5.934	96%	4.847	4.311	112%
	1:3	8.422	7.673	110%	1.438	1.477	97%	0.063	0.066	96%	2.976	2.967	100%	2.666	2.155	124%
	1:7	5.239	3.836	137%	1.228	0.738	166%	0.076	0.033	230%	2.358	1.484	159%	1.760	1.078	163%
II	1:0	6.031			4.195			0.490			5.640			3.017		
	1:1	2.510	3.016	83%	2.351	2.098	112%	0.262	0.245	107%	2.335	2.820	83%	1.517	1.509	101%
	1:3	1.497	1.508	99%	1.558	1.049	149%	0.174	0.122	142%	1.455	1.410	103%	0.995	0.754	132%
	1:7	1.092	0.754	145%	1.019	0.524	194%	0.112	0.061	183%	1.019	0.705	145%	0.704	0.377	187%
III	1:0	5.474			5.535			0.297			5.115			1.127		
	1:1	2.731	2.737	100%	2.657	2.767	96%	0.134	0.149	90%	2.476	2.557	97%	0.559	0.564	99%
	1:3	1.402	1.368	102%	1.431	1.384	103%	0.072	0.074	97%	1.321	1.279	103%	0.292	0.282	104%
	1:7	0.889	0.684	130%	0.936	0.692	135%	0.052	0.037	139%	0.819	0.639	128%	0.200	0.141	142%
IV	1:0	7.968			7.845			0.470			5.991			1.189		
	1:1	3.822	3.984	96%	4.237	3.923	108%	0.214	0.235	91%	2.869	2.996	96%	0.690	0.594	116%
	1:3	2.348	1.992	118%	2.871	1.961	146%	0.169	0.118	144%	1.772	1.498	118%	0.444	0.297	150%
	1:7	1.465	0.996	147%	1.867	0.981	190%	0.122	0.059	208%	1.287	0.749	172%	0.297	0.149	200%
V	1:0	12.961			14.087			1.467			14.260			2.098		
	1:1	6.285	6.481	97%	7.663	7.043	109%	0.732	0.733	100%	6.556	7.130	92%	1.167	1.049	111%
	1:3	3.021	3.240	93%	3.572	3.522	101%	0.332	0.367	90%	2.971	3.565	83%	0.678	0.524	129%
	1:7	2.447	1.620	151%	3.634	1.761	206%	0.284	0.183	155%	2.882	1.783	162%	0.513	0.262	196%

Table 13. (Continued.)

		stearic acid			gondoic acid			erucic acid			behenic acid			nervonic acid		
Animal	**Feces:Al_2O_3**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**
I	1:0	24.186			0.455			0.050			0.384			0.372		
	1:1	12.097	12.093	100%	0.234	0.227	103%	0.016	0.025	62%	0.223	0.192	116%	0.194	0.186	104%
	1:3	6.465	6.046	107%	0.123	0.114	108%	0.009	0.013	71%	0.134	0.096	140%	0.117	0.093	126%
	1:7	3.882	3.023	128%	0.079	0.057	138%	0.006	0.006	89%	0.104	0.048	217%	0.089	0.046	192%
II	1:0	3.105			0.174			0.041			0.198			0.347		
	1:1	1.259	1.552	81%	0.082	0.087	94%	0.016	0.020	76%	0.134	0.099	135%	0.183	0.173	105%
	1:3	0.713	0.776	92%	0.047	0.043	108%	0.007	0.010	65%	0.093	0.049	187%	0.117	0.087	135%
	1:7	0.503	0.388	130%	0.030	0.022	139%	0.004	0.005	80%	0.096	0.025	388%	0.105	0.043	242%
III	1:0	2.126			0.139			0.014			0.245			0.187		
	1:1	1.046	1.063	98%	0.073	0.070	105%	0.006	0.007	86%	0.155	0.122	127%	0.121	0.093	129%
	1:3	0.494	0.532	93%	0.030	0.035	86%	0.001	0.004	24%	0.098	0.061	161%	0.079	0.047	170%
	1:7	0.328	0.266	123%	0.019	0.017	109%	0.001	0.002	39%	0.083	0.031	272%	0.065	0.023	280%
IV	1:0	3.380			0.189			0.019			0.235			0.164		
	1:1	1.626	1.690	96%	0.088	0.095	93%	0.007	0.009	74%	0.164	0.118	139%	0.121	0.082	148%
	1:3	0.882	0.845	104%	0.062	0.047	130%	0.007	0.005	153%	0.115	0.059	196%	0.091	0.041	222%
	1:7	0.558	0.423	132%	0.037	0.024	158%	0.001	0.002	61%	0.088	0.029	301%	0.071	0.020	345%
V	1:0	8.891			0.488			0.035			0.307			0.131		
	1:1	4.277	4.446	96%	0.260	0.244	106%	0.024	0.018	136%	0.203	0.153	132%	0.113	0.066	171%
	1:3	2.071	2.223	93%	0.126	0.122	103%	0.020	0.009	225%	0.120	0.077	156%	0.072	0.033	219%
	1:7	1.479	1.111	133%	0.104	0.061	171%	0.009	0.004	196%	0.114	0.038	298%	0.078	0.016	475%

Table 13. (Continued.)

		cholesterol			brassicasterol			lathosterol			campesterol			stigmasterol		
Animal	**Feces:Al_2O_3**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**
I	1:0	6.705			0.014			0.040			1.223			0.467		
	1:1	3.253	3.352	97%	0.002	0.007	28%	0.011	0.020	53%	0.598	0.611	98%	0.223	0.234	95%
	1:3	1.788	1.676	107%	0.001	0.003	38%	0.006	0.010	63%	0.306	0.306	100%	0.109	0.117	93%
	1:7	0.991	0.838	118%	0.001	0.002	79%	0.005	0.005	95%	0.176	0.153	115%	0.057	0.058	98%
II	1:0	2.910			0.219			0.022			1.107			0.398		
	1:1	1.526	1.455	105%	0.099	0.109	90%	0.011	0.011	100%	0.555	0.553	100%	0.189	0.199	95%
	1:3	0.850	0.728	117%	0.053	0.055	96%	0.007	0.006	128%	0.314	0.277	113%	0.102	0.099	102%
	1:7	0.530	0.364	146%	0.032	0.027	117%	0.006	0.003	204%	0.216	0.138	156%	0.069	0.050	138%
III	1:0	2.172			0.049			0.015			1.891			0.691		
	1:1	0.967	1.086	89%	0.007	0.024	30%	0.008	0.007	105%	0.887	0.945	94%	0.325	0.346	94%
	1:3	0.409	0.543	75%	0.002	0.012	15%	0.005	0.004	128%	0.368	0.473	78%	0.130	0.173	75%
	1:7	0.254	0.272	93%	0.003	0.006	41%	0.004	0.002	202%	0.234	0.236	99%	0.075	0.086	87%
IV	1:0	2.318			0.041			0.016			1.679			0.705		
	1:1	1.174	1.159	101%	0.010	0.021	46%	0.009	0.008	118%	0.899	0.839	107%	0.384	0.352	109%
	1:3	0.630	0.579	109%	0.003	0.010	32%	0.006	0.004	159%	0.469	0.420	112%	0.192	0.176	109%
	1:7	0.302	0.290	104%	0.003	0.005	52%	0.004	0.002	222%	0.234	0.210	112%	0.094	0.088	107%
V	1:0	1.333			0.007			0.016			0.862			0.425		
	1:1	0.662	0.666	99%	0.003	0.004	87%	0.010	0.008	123%	0.456	0.431	106%	0.233	0.212	110%
	1:3	0.326	0.333	98%	0.001	0.002	70%	0.006	0.004	141%	0.223	0.216	103%	0.111	0.106	105%
	1:7	0.214	0.167	128%	0.001	0.001	166%	0.005	0.002	244%	0.155	0.108	144%	0.066	0.053	124%

Table 13. (Continued.)

		fucosterol			β-sitosterol			coprostanol			cholestanol			sitostanol		
Animal	**Feces:Al_2O_3**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**	**Obs.**	**Exp.**	**O/E**
I	1:0	0.100			2.034			0.025			0.091			0.549		
	1:1	0.051	0.050	102%	1.047	1.017	103%	0.018	0.013	141%	0.044	0.046	95%	0.266	0.275	97%
	1:3	0.028	0.025	111%	0.559	0.509	110%	0.012	0.006	189%	0.023	0.023	101%	0.133	0.137	97%
	1:7	0.017	0.013	135%	0.353	0.254	139%	0.011	0.003	355%	0.015	0.011	133%	0.076	0.069	111%
II	1:0	0.063			1.413			0.062			0.121			0.172		
	1:1	0.031	0.032	97%	0.751	0.707	106%	0.032	0.031	105%	0.055	0.060	92%	0.084	0.086	98%
	1:3	0.018	0.016	113%	0.441	0.353	125%	0.020	0.015	129%	0.031	0.030	103%	0.047	0.043	110%
	1:7	0.014	0.008	180%	0.344	0.177	195%	0.018	0.008	227%	0.021	0.015	142%	0.036	0.021	170%
III	1:0	0.167			3.404			0.538			0.171			0.972		
	1:1	0.081	0.084	97%	1.575	1.702	93%	0.202	0.269	75%	0.075	0.085	87%	0.443	0.486	91%
	1:3	0.034	0.042	81%	0.695	0.851	82%	0.089	0.135	66%	0.031	0.043	74%	0.181	0.243	75%
	1:7	0.022	0.021	106%	0.465	0.426	109%	0.056	0.067	84%	0.021	0.021	96%	0.117	0.121	96%
IV	1:0	0.167			2.886			0.048			0.149			0.860		
	1:1	0.089	0.083	107%	1.531	1.443	106%	0.028	0.024	117%	0.072	0.074	97%	0.443	0.430	103%
	1:3	0.045	0.042	108%	0.815	0.722	113%	0.019	0.012	153%	0.038	0.037	101%	0.222	0.215	103%
	1:7	0.024	0.021	116%	0.443	0.361	123%	0.013	0.006	222%	0.020	0.019	106%	0.113	0.107	105%
V	1:0	0.057			1.301			0.038			0.051			0.194		
	1:1	0.033	0.028	115%	0.746	0.651	115%	0.026	0.019	137%	0.028	0.025	110%	0.107	0.097	110%
	1:3	0.017	0.014	122%	0.389	0.325	120%	0.016	0.009	166%	0.015	0.013	116%	0.054	0.048	111%
	1:7	0.014	0.007	198%	0.303	0.163	186%	0.016	0.005	329%	0.012	0.006	190%	0.034	0.024	139%

Table 14. Spiking recovery of fatty acids demonstrated by pair-wise combinations of samples from four animals.

Sample Name	Weight 1 (mg)	Weight 2 (mg)	palmitic acid (µg/mg)	linoleic acid (µg/mg)	α-linolenic acid (µg/mg)	oleic acid (µg/mg)	*cis*-vaccenic acid (µg/mg)	stearic acid (µg/mg)	gondoic acid (µg/mg)	erucic acid (µg/mg)	behenic acid (µg/mg)	nervonic acid (µg/mg)
			Observed									
A	10.8	-	3.107	2.598	0.361	2.807	1.800	1.379	0.150	0.026	0.082	0.172
B	11.6	-	21.412	17.889	0.373	28.551	3.807	16.926	2.961	0.767	0.997	1.372
C	11.2	-	8.301	8.844	0.757	9.171	1.653	7.366	0.511	0.046	0.347	0.095
D	11.3	-	16.813	4.644	0.224	9.067	6.330	17.712	0.503	0.050	0.460	0.408
AB	6.2	5.9	14.858	11.020	0.464	20.483	2.457	10.669	1.925	0.438	0.565	0.824
AC	6.3	5.1	6.023	5.716	0.663	5.927	1.684	4.471	0.345	0.038	0.216	0.213
AD	6.1	6.1	10.336	3.009	0.212	5.398	4.124	10.293	0.323	0.035	0.264	0.282
BC	5.7	5.1	18.064	15.057	0.779	23.834	3.406	14.387	2.258	0.520	0.767	0.913
BD	6.0	5.4	18.744	12.340	0.513	21.928	5.398	16.742	2.144	0.466	0.779	0.915
CD	5.5	5.7	13.202	6.654	0.569	9.032	4.299	13.366	0.495	0.044	0.398	0.250
	Portion 1	Portion 2	Expected									
AB	0.51	0.49	12.020	10.043	0.366	15.348	2.771	8.954	1.520	0.387	0.528	0.757
AC	0.55	0.45	5.433	5.394	0.539	5.657	1.736	4.059	0.311	0.035	0.201	0.138
AD	0.50	0.50	9.957	3.619	0.292	5.935	4.064	9.544	0.326	0.038	0.271	0.290
BC	0.53	0.47	15.228	13.626	0.555	19.408	2.791	12.418	1.804	0.427	0.691	0.769
BD	0.53	0.47	19.237	11.627	0.303	19.339	5.000	17.297	1.799	0.428	0.743	0.917
CD	0.49	0.51	12.648	6.711	0.486	9.126	4.039	12.647	0.507	0.048	0.405	0.255
			O/E Ratios for mixed samples									
AB			81%	91%	79%	75%	113%	84%	79%	88%	93%	92%
AC			90%	94%	81%	95%	103%	91%	90%	92%	93%	65%
AD			96%	120%	138%	110%	99%	93%	101%	109%	103%	103%
BC			84%	90%	71%	81%	82%	86%	80%	82%	90%	84%
BD			103%	94%	59%	88%	93%	103%	84%	92%	95%	100%
CD			96%	101%	86%	101%	94%	95%	102%	110%	102%	102%
Median			**93%**	**94%**	**80%**	**92%**	**96%**	**92%**	**87%**	**92%**	**94%**	**96%**

Table 15. Spiking recovery of sterols demonstrated by pair-wise combinations of samples from four animals.

Sample Name	Weight 1 (mg)	Weight 2 (mg)	cholesterol (µg/mg)	brassicasterol (µg/mg)	lathosterol (µg/mg)	campesterol (µg/mg)	stigmasterol (µg/mg)	fucosterol (µg/mg)	β-sitosterol (µg/mg)	coprostanol (µg/mg)	cholestanol (µg/mg)	sitostanol (µg/mg)
			Observed									
A	10.8	-	1.001	0.138	0.024	0.609	0.140	0.030	1.002	0.018	0.050	0.023
B	11.6	-	5.609	0.021	0.032	0.156	0.085	0.017	0.177	0.018	0.132	0.007
C	11.2	-	1.533	0.027	0.030	0.831	0.393	0.054	1.404	0.051	0.071	0.190
D	11.3	-	7.957	0.025	0.040	1.295	0.437	0.098	2.485	0.031	0.134	0.639
AB	6.2	5.9	3.382	0.085	0.027	0.397	0.121	0.022	0.612	0.017	0.094	0.016
AC	6.3	5.1	1.333	0.108	0.026	0.797	0.293	0.044	1.343	0.037	0.066	0.110
AD	6.1	6.1	4.391	0.094	0.030	0.920	0.314	0.059	1.638	0.026	0.095	0.333
BC	5.7	5.1	4.055	0.027	0.034	0.532	0.266	0.038	0.823	0.040	0.116	0.110
BD	6.0	5.4	6.548	0.031	0.036	0.668	0.268	0.055	1.160	0.025	0.135	0.298
CD	5.5	5.7	4.779	0.049	0.034	1.041	0.436	0.076	1.890	0.048	0.106	0.416
	Portion 1	Portion 2	Expected									
AB	0.51	0.49	3.244	0.080	0.028	0.386	0.113	0.023	0.596	0.018	0.090	0.015
AC	0.55	0.45	1.240	0.088	0.027	0.709	0.253	0.040	1.183	0.032	0.059	0.098
AD	0.50	0.50	4.479	0.081	0.032	0.951	0.289	0.064	1.743	0.024	0.092	0.331
BC	0.53	0.47	3.686	0.024	0.031	0.475	0.231	0.034	0.758	0.033	0.103	0.094
BD	0.53	0.47	6.719	0.023	0.036	0.694	0.252	0.055	1.268	0.024	0.133	0.306
CD	0.49	0.51	4.810	0.026	0.035	1.068	0.416	0.076	1.957	0.040	0.103	0.419
			O/E Ratios for mixed samples									
AB			96%	94%	104%	97%	93%	105%	97%	102%	96%	94%
AC			93%	82%	101%	89%	86%	92%	88%	88%	91%	89%
AD			102%	86%	107%	103%	92%	109%	106%	91%	97%	100%
BC			91%	87%	92%	89%	87%	90%	92%	83%	89%	85%
BD			103%	74%	100%	104%	94%	100%	109%	94%	99%	102%
CD			101%	53%	104%	103%	95%	101%	104%	84%	97%	101%
Median			**98%**	**84%**	**102%**	**100%**	**92%**	**100%**	**100%**	**90%**	**97%**	**97%**

Table 16. Precision and reproducibility of the assay. [a]Concentrations of samples used (the median and range of the median of six replicates from six different animals). [b]Coefficient of variation, calculated from six replicates from each of six animals.

	intra-assay		**inter-assay**	
Compound	**median [range] µg/mg feces[a]**	**median [range] %CV[b]**	**median [range] µg/mg feces[a]**	**median [range] %CV[b]**
palmitic acid	3.63 [1.8-11.62]	4 [2-8] %	3.38 [2.35-11.18]	7 [5-14] %
linoleic acid	2.83 [0.92-7.59]	8 [4-18] %	2.73 [1.15-7.55]	10 [4-14] %
α-linolenic acid	0.16 [0.12-0.59]	14 [7-34] %	0.27 [0.11-0.61]	12 [10-79] %
oleic acid	2.79 [0.87-8.34]	4 [4-11] %	2.92 [0.88-8.2]	9 [4-13] %
cis-vaccenic acid	0.77 [0.25-3.46]	5 [2-7] %	0.81 [0.28-1.82]	9 [4-12] %
stearic acid	2.99 [1.41-10.57]	4 [2-7] %	2.07 [1.46-6.2]	6 [5-12] %
arachidonic acid	0.72 [0.36-2.37]	6 [3-7] %	0.57 [0.32-2.24]	11 [5-21] %
gondoic acid	0.15 [0.08-0.36]	5 [3-9] %	0.16 [0.06-0.36]	11 [9-18] %
erucic acid	0.03 [0.02-0.06]	10 [3-47] %	0.03 [0.02-0.07]	18 [12-62] %
behenic acid	0.32 [0.22-0.94]	6 [2-9] %	0.29 [0.13-0.97]	12 [7-16] %
nervonic acid	0.11 [0.09-0.38]	8 [4-11] %	0.13 [0.07-0.46]	17 [8-23] %
cholesterol	3.03 [2.01-12.84]	4 [1-7] %	2.66 [2.01-4.5]	6 [4-15] %
brassicasterol	0.07 [0.04-0.32]	4 [2-5] %	0.1 [0.04-0.28]	9 [6-11] %
lathosterol	0.04 [0.03-0.08]	5 [3-6] %	0.04 [0.03-0.06]	8 [5-9] %
campesterol	1.07 [0.43-2.19]	4 [2-6] %	1.1 [0.44-1.97]	6 [5-10] %
stigmasterol	0.4 [0.15-0.73]	4 [2-8] %	0.4 [0.16-0.6]	7 [5-13] %
fucosterol	0.07 [0.03-0.15]	6 [3-9] %	0.07 [0.03-0.11]	10 [7-16] %
β-sitosterol	1.63 [0.78-3.47]	4 [2-7] %	1.44 [0.82-2.31]	6 [5-10] %
coprostanol	0.03 [0.01-0.16]	5 [3-17] %	0.01 [0.01-0.1]	11 [3-16] %
cholestanol	0.12 [0.07-0.3]	4 [1-5] %	0.12 [0.07-0.3]	9 [5-11] %
sitostanol	0.08 [0.01-0.49]	4 [1-6] %	0.07 [0.01-0.24]	11 [6-15] %

6.4 DISCUSSION

The assay described here quantifies eleven fatty acids and ten sterols. In comparison to serum samples, the potential range of concentrations for analytes in feces is expected to be much wider and more variable between individuals. It also may be expected that feces are more easily influenced by diet, but for exploring the effects of gastrointestinal diseases, it is biologically the most relevant sample for its relationship with the GI tract and the microbiota.

Although the entire range of concentrations for all compounds may not fall within the limits of quantitation, analytes in the vast majority of samples do fall within the range of quantification for this assay. Sensitivity for some compounds would likely be improved if the split ratio for the injection were decreased, but for other compounds this might saturate the detector and result in poor quantification. Considering the inherent variability associated with a heterogeneous mixture such as fecal matter, the validation parameters are considered satisfactory. It is likely that homogenization and coarse filtration prior to lyophilization would improve the reproducibility of the assay, but the improvement may not be clinically relevant. On the other hand, aliquots for assessment of inter-assay variability were taken after lyophilization, so it is worth considering that some additional variability could occur during the lyophilization step and this has not been captured by this validation of the assay.

The method used for dilutional parallelism in this validation is somewhat unconventional. While serum can easily be diluted with water, dilution of feces prior to lyophilization would not have affected the dry weight. Therefore, an inert, dry substance that could dilute the lyophilized feces on a mass-basis was required. In hindsight, it potentially should be tested if the presence of aluminum oxide has any inherent effect on the results, though this is unlikely considering the consistency of recoveries.

There are some additional limitations and remaining considerations. First, the characterization of compounds was based on authentic standards, prepared and analyzed in isolation. In fecal samples, peaks with the appropriate fragmentation spectra and retention times were considered positively identified. However, closely-related compounds may still co-elute and produce similar spectra, so it would be naïve to exclude the possibility that peaks in a highly complex sample matrix such as fecal matter may in fact be combinations of isomers. This is particularly likely for fatty acids, where the fragmentation spectra for two isomers may be nearly identical.

Another factor that was not specifically evaluated is the robustness of the assay. Although there are several individuals trained to perform this assay, the validation parameters reported here were based on analyses done exclusively by one person. The use of the LCS and other quality control samples does support that batches prepared by different individuals do show generally reproducible results, but this was not explicitly assessed in this study. In addition, the tolerance for preparation variability (e.g., intentionally varying quantities of reagents from the stipulated amounts, changes to temperatures, etc.) was not tested. The confidence in the assay would be strengthened by exploring what degree of changes could be tolerated without significantly changing the resultant values.

Beyond technical considerations, there are concerns pertaining to normal physiology, including day-to-day variation, or the effect of a single bolus of dietary fat (e.g., a treat). Also, stability of the samples has not yet been tested. Although the literature reports using sterols in soil and sediment to identify ancient bathrooms in archaeological excavations, suggesting a good degree of stability, this is far from quantitative. Since fecal matter is a biologically and chemically active matrix, it would not be surprising if the concentrations of metabolites were to change if not appropriately preserved.

Another item for further consideration is the presence of sterol esters. An alternative method might include a hydrolysis step prior to butyl esterification in order to liberate sterols and fatty acids from their esterified forms (including triacylglycerides and phospholipids) (Keller and Jahreis 2004).

6.5 CONCLUSIONS

The FASter assay using gas chromatography-mass spectrometry was shown to be precise, reproducible, and sensitive for the quantification of several sterols and fatty acids in canine fecal samples.

7. FECAL CONCENTRATIONS OF STEROLS AND FATTY ACIDS IN DOGS WITH CHRONIC ENTEROPATHY

OVERVIEW

Diseases of chronic inflammation of the gastrointestinal tract, including idiopathic inflammatory bowel disease (IBD), are thought to be perpetuated by bacterial dysbiosis and dysregulation of the mucosal immune system in both humans and animals. Gastrointestinal (GI) absorption of metabolites is altered by inflammation, with concurrent changes in microbial metabolism within the GI tract. Cholesterol is the primary sterol in mammals; it is incorporated into cell membranes throughout the body and used as a building block for endogenous hormones, steroids, and bile acids. Some phytosterols have been shown to have anti-inflammatory properties and have been shown to decrease disease severity in a murine colitis model. Therefore, the aim of this study was to compare fecal sterol profiles of dogs with chronic enteropathy to those of healthy control dogs.

Fecal samples were collected from dogs with chronic enteropathy (n=44) and from healthy control dogs (n=13). Sterols in lyophilized feces were subjected to trimethylsilyl ether derivatization and analyzed by gas chromatography-mass spectrometry (GC-MS) operating in selected ion monitoring (SIM) mode. Target analytes included cholesterol, cholestanol, β-sitosterol, sitostanol, fucosterol, stigmasterol, and campesterol. Fecal concentrations of sterols were expressed as μg/mg of lyophilized feces. A Mann-Whitney U test was used for comparison between animal groups, and a Benjamini-Hochberg step-up method was used to correct for multiple comparisons. Statistical significance was set at $p<0.05$.

Fecal cholesterol and cholestanol were not significantly altered between healthy dogs and dogs with chronic enteropathy. However, β-sitosterol and sitostanol were significantly decreased

in dogs with chronic enteropathy, and campesterol and fucosterol were also significantly decreased prior to adjusting *p*-values for multiple comparisons.

In conclusion, this study suggests that the fecal sterol profile in dogs with chronic enteropathy is characterized by decreased phytosterols. Additional studies are required to explore the effect of dietary supplementation with phytosterols and to investigate how these sterols are related to specific bacterial groups that digest dietary plant material in these patients.

7.1 INTRODUCTION

The importance of sterols and fatty acids in host metabolism has been described in Section 1. Aberrant lipid metabolism has been implicated to play a role in the pathogenesis of inflammatory bowel disease (IBD) in people (Shores et al. 2011). Gene expression studies on duodenal mucosal biopsies from dogs identified several genes that were differentially expressed between dogs with IBD and healthy control dogs, and several of those altered genes are relevant to lipid metabolism (Wilke et al. 2012). Examples include a 92-fold decrease in the gene encoding neurotensin (NTS), which in humans has been linked to obesity (J. Li et al. 2016) due to its role in fatty acid absorption. Wilke et al. (2012) also found a nearly 2-fold downregulation of the gene encoding fatty acid synthase (FASN), a 14-fold decrease in fatty acid binding protein 6 (FABP6, also known as gastrotropin), and a 4-fold increase in peroxisome proliferator-activated receptor gamma (PPARG), which regulates fatty acid storage and glucose metabolism. Another study using duodenal mucosal biopsies concluded that polyunsaturated fatty acid-enriched diets affected genes of cholesterol homeostasis, finding that caveolin-1, ABCA1, and SREBP-2 were downregulated in dogs with IBD on the PUFA-enriched diet (but this change was not observed for dogs with food-responsive diarrhea, and the diets were comprised of different ingredients) (Ontsouka et al. 2010).

Based on the altered gene expression demonstrated in these studies pertaining to absorption and metabolism of lipids, it would be anticipated to find altered concentrations of lipids in the feces of dogs with chronic GI inflammation. Previous untargeted metabolomics studies (see Section 5) further supported that abundances of lipid metabolites are altered in dogs with chronic enteropathy, finding relative changes specifically among fatty acids and sterols. It was hypothesized that an analysis of a new cohort of dogs with chronic enteropathy would show quantitatively altered fecal sterol and fatty acid concentrations compared to healthy control dogs. The current study aimed to use the FASter assay (described in Section 6) as a targeted metabolomics panel to quantify eleven fecal fatty acids and ten sterols in dogs with chronic enteropathy compared to healthy control dogs, using the analyte results collectively to define an overall fecal FASter profile.

7.2 MATERIALS AND METHODS

7.2.1 Study population and sample collection

Naturally-passed fecal samples were retrospectively selected from surplus samples that had been stored at -80°C. Included were healthy control dogs (n=13) that had undergone physical examination as well as bloodwork to verify health status, and a corresponding owner questionnaire had asserted no recent (> 8 weeks) antibiotic usage. Inclusion criteria for the diseased samples (n=44) was availability of a surplus fecal sample, no recent antibiotics usage reported in the medical history, tentative diagnosis of IBD with active disease at the time of collection, and fecal sample collection prior to bowel cleansing (if such had been done for the purpose of upper or lower endoscopy).

7.2.2 Sample preparation and instrument analysis

Samples were prepared and analyzed by the FASter assay using gas chromatography-mass spectrometry (GC-MS) as described in Section 6.2.

7.2.3 Statistical analyses

Results in μg/mg lyophilized feces for all samples were processed in JMP (SAS, Durham, NC, USA) using Mann-Whitney *U* test to identify significantly altered fatty acids and sterols. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg step-up method and significance was defined as a corrected $p<0.05$.

Based on the physiological relationship between phytosterols and cholesterol, meaning the nonspecific absorption of sterols via NPC1L1 with selective expulsion of non-cholesterol by ABCG5/G8, a ratio of phytosterols (i.e., brassicasterol, fucosterol, campesterol, β-sitosterol, sitostanol, and stigmasterol) to cholesterol was calculated. The ratio of stanols (i.e., coprostanol, cholestanol, and sitostanol) to Δ5 sterols (i.e., cholesterol, β-sitosterol, campesterol, stigmasterol, and fucosterol) was also calculated. These ratios were compared between groups using Mann-Whitney *U* tests with statistical significance set at as $p<0.05$.

Finally, data were visually analyzed from a multivariate perspective using MetaboAnalyst (Xia et al. 2015; Xia and Wishart 2011, 2016). Raw concentrations were log-transformed and Pareto-scaled to achieve a more Gaussian distribution, then used to generate PCA and PLS-DA plots reflecting the distribution of samples by their collective FASter composition profiles. MetaboAnalyst was also used to generate a Pearson correlation matrix between metabolites.

7.3 RESULTS

Before correcting for multiple comparisons, ten of the twenty-one metabolites were potentially significant, but only three remained significant after correcting for multiple

comparisons: arachidonic acid, β-sitosterol, and sitostanol. A summary of the results for all compounds (group medians and ranges, with corresponding *p*- and *q*-values) is given in Table 17.

The ratio of phytosterols to cholesterol was also significantly altered between groups. The median [range] for healthy animals was 2.398 [1.090-3.669] while for the diseased group it was only 0.682 [0.019-5.106], (*p*=0.0006). The ratio of stanols to sterols was also decreased in samples from dogs with IBD. The median [range] for healthy animals was 0.129 [0.073-0.205] while for the diseased group it was only 0.056 [0.009-0.165], (*p*=0.0002).

PCA and PLS-DA plots showing the distribution of samples when their composition is considered from a multivariate perspective (supervised and unsupervised, respectively) are shown in Figure 25, and the correlation matrix is shown in Figure 26.

Table 17. Concentrations of fatty acids and sterols in dogs with IBD compared to healthy control dogs.

	Healthy		IBD			
Compound	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
palmitic acid (16:0) (μg/mg)	4.936	[2.753-6.639]	5.037	[0.823-30.053]	0.7107	0.7537
linoleic acid (18:2 n-6) (μg/mg)	4.576	[1.382-14.933]	3.707	[0.202-56.091]	0.4189	0.5498
α-linolenic acid (18:3 n-3) (μg/mg)	0.356	[0.113-0.679]	0.491	[0.042-7.342]	0.2659	0.4295
oleic acid (18:1 n-9) (μg/mg)	4.721	[1.892-7.708]	4.657	[0.322-57.061]	0.8121	0.8121
cis-vaccenic acid (18:1 n-7) (μg/mg)	0.806	[0.583-2.223]	1.139	[0.152-11.271]	0.5491	0.6783
stearic acid (18:0) (μg/mg)	1.894	[1.195-3.684]	3.840	[0.41-40.124]	0.0980	0.1871
arachidonic acid (20:4 n-6) (μg/mg)	0.341	[0.19-0.484]	0.734	[0.188-7.315]	**0.0000**	**0.0003**
gondoic acid (20:1 n-9) (μg/mg)	0.184	[0.115-0.279]	0.232	[0.021-1.969]	0.3273	0.4910
erucic acid (22:1 n-9) (μg/mg)	0.029	[0.019-0.047]	0.044	[0.013-0.238]	**0.0260**	0.0890
behenic acid (22:0) (μg/mg)	0.223	[0.154-0.391]	0.291	[0.075-1.837]	**0.0382**	0.0890
nervonic acid (24:1 n-9) (μg/mg)	0.173	[0.107-0.384]	0.249	[0.059-0.965]	**0.0458**	0.0962
cholesterol (μg/mg)	1.860	[1.182-5.67]	3.749	[0.648-17.46]	**0.0324**	0.0890
brassicasterol (μg/mg)	0.031	[0.021-0.16]	0.046	[0.002-0.377]	0.6755	0.7537
lathosterol (μg/mg)	0.032	[0.02-0.068]	0.035	[0.019-0.108]	0.1534	0.2685
campesterol (μg/mg)	1.284	[0.739-2.422]	0.922	[0.163-2.672]	**0.0373**	0.0890
stigmasterol (μg/mg)	0.465	[0.297-0.765]	0.429	[0.005-1.019]	0.3563	0.4988
fucosterol (μg/mg)	0.117	[0.077-0.233]	0.067	[0.012-0.672]	**0.0179**	0.0890
β-sitosterol (μg/mg)	2.012	[1.354-4.42]	1.243	[0.086-5.989]	**0.0008**	**0.0055**
coprostanol (μg/mg)	0.087	[0.025-0.607]	0.035	[0.008-1.494]	**0.0280**	0.0890
cholestanol (μg/mg)	0.182	[0.071-0.473]	0.207	[0.032-0.746]	0.7178	0.7537
sitostanol (μg/mg)	0.594	[0.207-1.253]	0.083	[0.006-1.236]	**0.0000**	**0.0003**

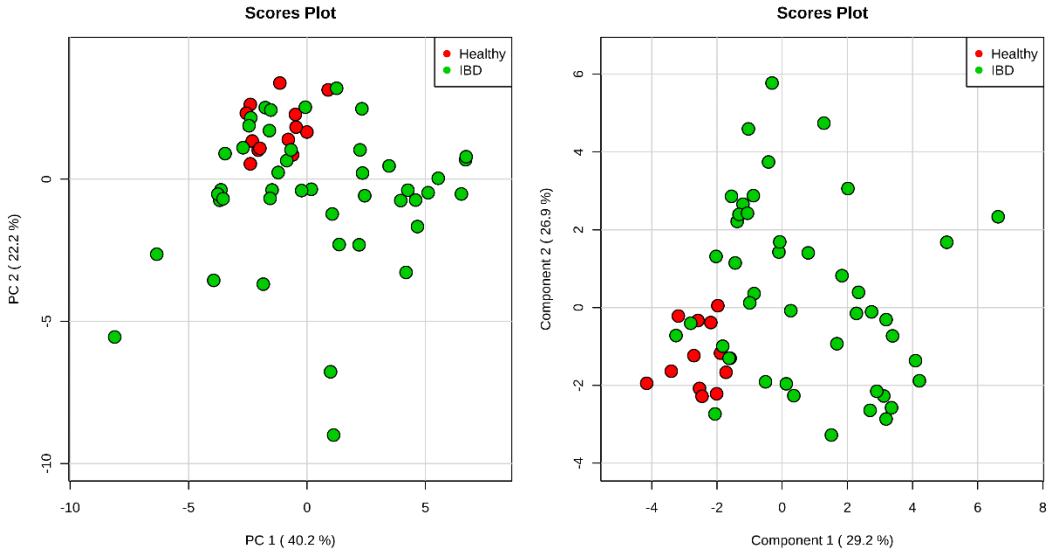


Figure 25. PCA (left) and PLS-DA (right) plots showing slightly improved separation using the supervised method.

7.4 DISCUSSION

The results of this study are supportive of the hypothesis that lipid metabolism is affected in dogs with IBD. However, determining the cause and effect relationship for such a change is much more challenging, likely requiring prospective experiments under better controlled conditions, such as a standardized diet, and unlikely to be possible using client-owned animals with spontaneous disease. One of the interesting findings in this study is the strong association between fecal arachidonic acid and disease. Arachidonic acid is considered the precursor to pro-inflammatory compounds, including leukotrienes with chemotactic properties for neutrophils (Samuelsson 1991). More recently, phospholipids containing arachidonic acid have also been associated with reactive microglia (macrophages specifically in the brain) after spinal cord injury (D. Xu et al. 2016), underscoring the potentially critical role that arachidonic acid plays in immune homeostasis. Unfortunately, it is difficult to interpret what increased concentrations in the feces

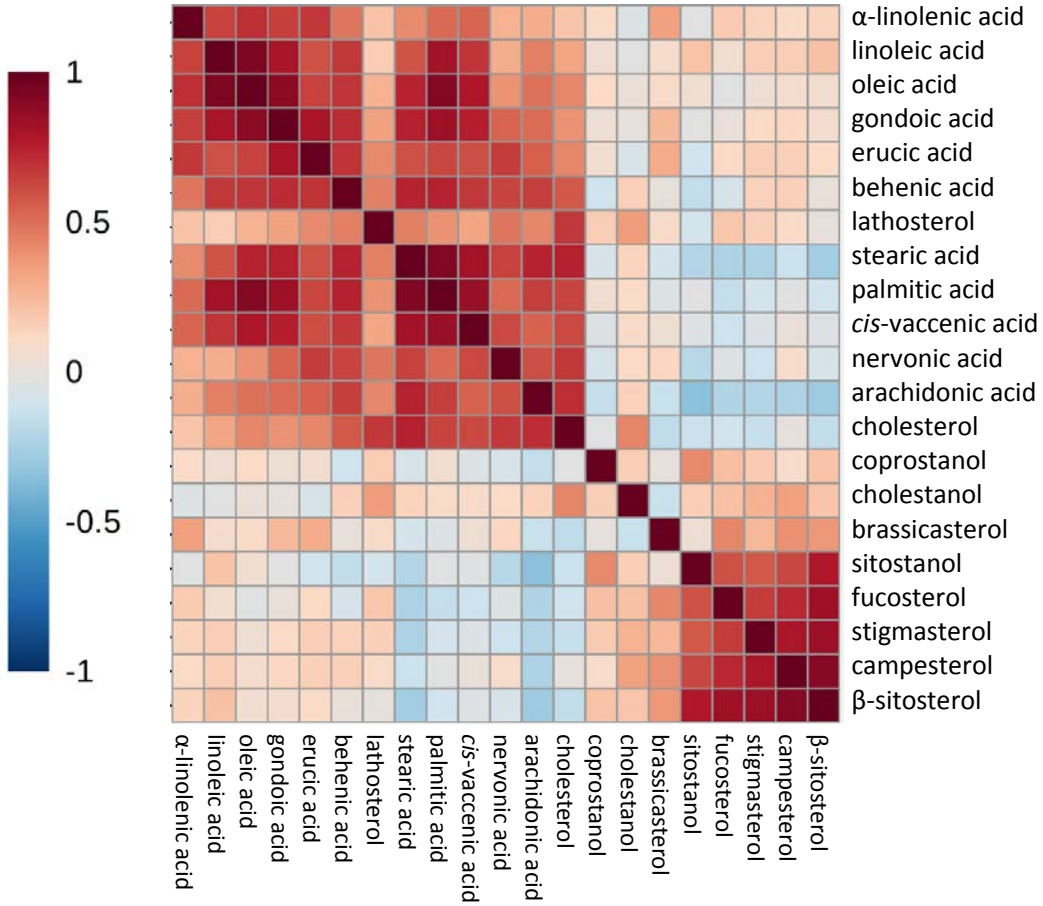


Figure 26. Correlation matrix plot showing relationships between metabolites across all samples, irrespective of sample classification. The color scale indicates metabolites that positively correlate as red, and metabolites that negatively correlate as blue, based on Pearson rho.

may mean for the diseased dogs in this study. Since feces are a means for elimination from the body, higher concentrations could reflect efficient excretion of this bioactive material. On the other hand, the bioactivity of the compound may result in perpetuation of inflammation.

Other fatty acids that tended to be altered but did not quite attain significance in this study included erucic, behenic, and nervonic acids, which are all considered very long chain fatty acids (VLCFAs). Accumulation of VLCFAs was observed also in the untargeted metabolomics study described in Section 5, and may in part be related to the 1.4- to 1.9-fold upregulation of LCFA

elongation enzyme genes (ELOVL5 and ELOVL7) observed by Wilke et al. (2012). There is a review by Anderson and Stahl (2013) regarding the solute carrier family 27 proteins, which are fatty acid transporters. Member 2 of this family (SLC27A2, encoding a very long chain acyl-CoA synthetase) was 4-fold downregulated in the gene expression study by Wilke et al. (2012), yet member 6 (SLC27A6, also known as FATP6) was 3-fold upregulated. SLC27A6 has been suggested as a predominant fatty acid transporter in the heart and SLC27A2 was found primarily in the liver and kidney in rats (Anderson and Stahl 2013). SLC27A2 also may be involved in bile acid synthesis, specifically activating the precursor of cholic acid by forming the coenzyme-A derivative. In conjunction, fatty acid binding protein 6 (FABP6, not to be confused with FATP6) was nearly 14-fold downregulated in the study by Wilke et al. (2012), and this protein is thought to bind both fatty acids and bile acids in the ileum, its predominant location. Interestingly, FABP2 (also called intestinal-type fatty acid binding protein, I-FABP) was not reported in the gene expression study, but plasma levels have been reported to correlate to mucosal injury in an ischemia-reperfusion model (Schellekens et al. 2014). The relevance of these gene expression alterations in the duodenum of the dog is difficult to interpret, but certainly suggests there is much still to discover about these fatty acid transport and binding proteins and their role in gastrointestinal physiology and disease pathogenesis. Unfortunately, the fatty acids quantified in this assay include only one monounsaturated *n*-3 fatty acid, α-linolenic acid, so the correlations between *n*-3 and *n*-6 fatty acids cannot be discerned with much certainty from our data. There is active research exploring how dietary PUFAs may modify overall fatty acid distribution and bioavailability (Q. Yang et al. 2017) and some research specifically looking at PUFAs for treating canine chronic enteropathy (Ontsouka et al. 2010).

Additional significant changes in concentrations were observed among the sterols. Sitostanol has consistently been strongly associated with health in the current study and the

untargeted metabolomics study, but there does not appear to be much literature regarding this phenomenon in people. Instead, sitostanol apparently has been appreciated as a nonabsorbable compound to use as a normalization factor for absorption of other lipids (Hassan and Rampone 1979). Abnormal absorption of other phytosterols due to genetic disorders is also known in humans (Tang et al. 2009; Yoo 2016), but again, changes in fecal concentrations of phytosterols associated with disease appears to be a relatively novel subject. Therefore, the cause of decreased phytosterol concentrations (in this study, most notably β-sitosterol and sitostanol) in feces of dogs with IBD can only be speculated upon, with very little information available to either validate or refute.

The origin of phytosterols in feces is presumed to be diet, but the physiology of the gastrointestinal absorption of sterols modifies their concentrations. As shown in Section 3, phytosterols represent a small portion of the total quantified lipids in duodenal samples from healthy dogs, and an apparent increase in concentration distally could be ascribed to the depletion of other materials from the lumen (i.e., normal absorption of nutrients). Under physiologic conditions, the intact intestinal mucosa would be expected to absorb both phytosterols and cholesterol via the NPC1L1 transporter. Cholesterol then should be processed for use (esterified into cholesteryl esters for storage, packed into chylomicrons for secretion via lacteals, or incorporated into the cell membrane) while phytosterols should be expelled back into the lumen via the ABCG5 and ABCG8 transporters. Excess cholesterol from the tissues (and presumably the small proportion of phytosterols that escaped expulsion at the level of the enterocytes) undergoes reverse cholesterol transport (RCT). This process employs the ABCA1 transporter to transfer cholesterol in peripheral tissues to high density lipoprotein (HDL), which then travels in the blood to the liver (Tall 1998; Brufau et al. 2011). Interestingly the ratio of LDL to HDL in humans is inverted compared to the dog, with nearly 87% of lipoprotein partitioned into HDL and only 11% as LDL in the dog, yet the total amount of lipid in circulation is similar between humans and dogs

(Maldonado et al. 2001). Hepatic cholesterol can then be disposed of directly in the bile, or synthesized into bile acids.

Hypocholesterolemia is not an unusual clinical finding among dogs or humans with IBD (Simpson and Jergens 2011; Agouridis et al. 2011; Hrabovsky et al. 2009), though the exact mechanism by which this occurs is unknown. A search of the literature for research related to fecal cholesterol did not yield articles relevant to inflammatory bowel disease in any species or experimental model of IBD. However, it would not seem unwise to connect the tendency towards hypocholesterolemia in both humans and dogs with IBD and the finding in this study for a trend towards increased fecal cholesterol in dogs with IBD. Analyses of serum in people with active Crohn's disease (n=24) revealed a nonsignificant decrease of sitosterol and squalene, and significant decreases of lathosterol and campesterol accompanying hypocholesterolemia relative to healthy control subjects (Hrabovsky et al. 2009). Although interpretation for elucidating a mechanism at the level of the intestinal mucosa is not possible, it does suggest that the mechanism at play results in both decreased phytosterols and cholesterol, rather than a loss of selectivity; yet this is not entirely in accordance with the observation in this study that the ratio of phytosterols to cholesterol was significantly decreased in dogs with IBD compared to healthy control dogs.

If the normal host physiology is considered to affect concentrations of fecal metabolites, the microbiota certainly also must be considered. Although the relevance of plant physiology to mammals may be questioned, Griebel and Zeier (2010) demonstrated that inoculation of the plant *Arabidopsis thaliana* with the bacterial pathogen *Pseudomonas syringae* induced the plant to convert β-sitosterol to stigmasterol (structurally identical except for an additional desaturation at C22 in stigmasterol), and furthermore that this resulted in enhanced susceptibility to *P. syringae*. A means of more direct metabolic interaction by the microbiota may be evidenced by the conversion of cholesterol to coprostanol occurring in the GI tract of humans (Antharam et al. 2016;

Gerard 2013; Gerard et al. 2007; Veiga et al. 2005; Olejníková et al. 2017). Chemically this conversion represents the hydrogenation of the C5 bond of cholesterol to form the fully saturated coprostanol. Interestingly, β-sitosterol is the C5-unsaturated analog of sitostanol, which strongly suggests the possibility of a similar mechanism (e.g., microbiota-mediated) for conversion of β-sitosterol to sitostanol as from cholesterol to coprostanol. Both represent the reduction of the Δ5 sterol to form the corresponding Δ0 stanol, and the microbial biohydrogenation of Δ5 sterols is known (Eyssen et al. 1973). The association of GI health with being a "high converter" of cholesterol-to-coprostanol has been shown in people (Antharam et al. 2016), so perhaps the observation of a significant increase in the Δ0/Δ5 ratio in feces of dogs with IBD is representative of a similar microbial etiology.

Regardless of the mechanism that underlies the difference in phytosterol concentrations, there is evidence that phytosterols may represent some therapeutic options. Aldini et al. (2014) showed that a two-week period of feeding a phytosterol-enriched diet to mice resulted in decreased severity and duration of dextran sodium sulfate (DSS)-induced colitis. The change in membrane fluidity induced by incorporation of sterols into membranes has previously been described in Section 1.6, and the relative effect of phytosterols versus cholesterol to membranes has also been reviewed (Dufourc 2008). Considering the underlying etiology of canine IBD is most certainly not oral intake of a detergent to disrupt cell membranes, perhaps this indicates that increased phytosterol concentrations exert a protective effect from whatever insult potentially incites IBD. However, aside from the suspicion that dog owners would happily purchase "Fidosterols" to protect their dogs from IBD, these relationships are merely ideas that require significantly more analysis before experimentation *in vivo*. Furthermore, a larger population of healthy animals should be sampled to develop a reference interval.

7.5 CONCLUSIONS

This study has demonstrated that lipid metabolism is affected in dogs with IBD, resulting in altered concentrations of sterols (sitostanol and β-sitosterol) and fatty acids (arachidonic acid), as well as changes in the ratio between phytosterols and cholesterol, and the ratio between saturated and C5-unsaturated sterols (i.e., coprostanol to cholesterol, and sitostanol to β-sitosterol).

8. LONGITUDINAL ASSESSMENT OF FECAL CONCENTRATIONS OF STEROLS AND FATTY ACIDS IN DOGS WITH CHRONIC AND ACUTE GASTROINTESTINAL DISEASES

8.1 INTRODUCTION

Sterols and fatty acids have been found to be altered in the feces of dogs with IBD compared to healthy control dogs, both by untargeted (Section 5) and targeted (Section 7) metabolomics studies. The biological and physiological relevance of these lipids to host health and in the context of canine IBD have been discussed previously. However, from the results of previous studies, two very broad questions immediately arose. First, are the observed changes in the fecal FASter profile specific to chronic gastrointestinal diseases, or will similar changes be observed in severe, acute disease, such as acute hemorrhagic diarrhea syndrome (AHDS)? Secondly, will the concentrations of these metabolites change over time? We hypothesized that different patterns of fecal sterols and fatty acids would be associated with different gastrointestinal disease phenotypes, and that these concentrations would vary over time with treatment. To investigate these hypotheses, we aimed to compare the fecal FASter profiles over time from dogs with chronic enteropathy (CE) and also from dogs with AHDS to those from healthy control dogs.

8.2 MATERIALS AND METHODS

8.2.1 Study population and sample collection

Naturally-passed fecal samples were retrospectively selected from available surplus samples from other studies, stored at -80°C. Samples from diseased dogs were categorized based on the diagnosis by the referring clinician. Baseline fecal samples were available from dogs with

acute hemorrhagic diarrhea syndrome (*n*=21), and follow up samples were available for a subset of these dogs collected on day 3 (*n*=10), day 7 (*n*=12), day 14 (*n*=11), day 21 (*n*=8), and day 90 (*n*=9). Baseline fecal samples were available from dogs diagnosed with idiopathic inflammatory bowel disease (*n*=32). For a cohort of these dogs that clinically improved with treatment (*n*=16), samples were also available from day 21 (*n*=11), day 56 (*n*=11), and > 1 year (*n*=7) follow up. The diagnosis of idiopathic IBD was based on lack of response to at least one dietary trial, though the specific diet attempted for management varied based on preferences and requirements from the clinician, patient, and owner. Naturally passed fecal samples from healthy control dogs were also collected (*n*=18). Assessment of health was based on owner questionnaire indicating no recent antibiotic usage and no clinical signs of gastrointestinal disease.

8.2.2 Sample preparation and instrument analysis

Samples were prepared and analyzed by the FASter assay using gas chromatography-mass spectrometry (GC-MS) as described in Section 6.2.

8.2.3 Statistical analyses

Results in μg/mg lyophilized feces for all samples were analyzed in JMP (SAS, Durham, NC, USA). Kruskal-Wallis and Benjamini-Hochberg step-up method to adjust for multiple comparisons were used to identify overall significantly altered compounds among healthy controls, AHDS (baseline), AHDS (day 90), IBD (baseline), and IBD (>1 year). A Dunn's post test was used to identify which pairwise comparisons were significantly different. Statistical significance was set at $p<0.05$. Multivariate evaluation of groups at baseline and the shifting composition over time was performed using MetaboAnalyst (Xia et al. 2015; Xia and Wishart 2011, 2016). Raw concentrations were log-transformed and Pareto-scaled to achieve a more Gaussian distribution. For baseline samples, a plot was generated of the Pearson correlation matrix

for metabolites (across both diseases and in health), along with a PCA plot. For the samples from dogs with AHDS, a PCA plot and a heatmap were generated to show the distribution of samples by their collective FASter composition profiles over time. For samples from dogs with IBD, a PCA plot was generated to show sample groups over time.

8.3 RESULTS

8.3.1 Univariate analysis

The summary for univariate analysis is shown in Table 18. Nearly all metabolites were significantly altered between at least one pairwise comparison; exceptions were oleic and palmitic acids. The baseline samples for dogs with AHDS were most often distinct from the other groups.

8.3.2 Multivariate analysis

8.3.2.1 Baseline profiles

The Pearson correlation matrix for all baseline samples is shown in Figure 27, and Figure 28 shows the PCA plot at baseline for the fecal FASter profile of dogs with AHDS, IBD, and healthy samples.

8.3.2.2 AHDS over time

The PCA plot showing the samples collected at each time point is shown in Figure 29. Samples are shown as dots; shaded areas represent the 95% confidence area for the group.

The heatmap for the samples is shown in Figure 30. Individual sterols and fatty acids are in rows, and samples are in columns. The color of each cell is relative to the normalized concentration, with high concentrations in red and low concentrations in blue.

Table 18. Summary of fatty acid and sterol concentrations at baseline and last time point for dogs with AHDS or IBD compared to healthy control dogs. Significant pairwise comparisons are indicated by not sharing a superscript letter.

	Healthy		AHDS day 0		AHDS day 90		IBD day 0		IBD > 1 year			
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
Compound	(µg/mg)		(µg/mg)		(µg/mg)		(µg/mg)		(µg/mg)			
Fatty Acids												
α-linolenic acid	0.321 [a]	[0.113-0.679]	0.096 [b]	[0.064-2.6]	0.133	[0.089-0.787]	0.491 [a]	[0.08-7.342]	0.278	[0.122-1.021]	**0.0000**	**0.0000**
arachidonic acid	0.357 [a]	[0.19-0.699]	3.372 [b]	[0.74-10.792]	0.799 [a,c]	[0.324-2.385]	0.705 [c]	[0.188-4.977]	0.608 [a,c]	[0.399-1.361]	**0.0000**	**0.0000**
behenic acid	0.238 [a]	[0.154-0.391]	0.471 [b]	[0.257-0.827]	0.213 [a]	[0.135-0.513]	0.300	[0.153-1.837]	0.262	[0.224-0.599]	**0.0001**	**0.0001**
cis-vaccenic acid	0.864 [a]	[0.544-2.223]	2.991 [b]	[1.078-8.829]	0.779 [a]	[0.464-2.158]	1.010 [a]	[0.215-11.271]	0.638 [a]	[0.307-4.428]	**0.0003**	**0.0003**
erucic acid	0.029 [a]	[0.019-0.047]	0.074 [b]	[0.039-0.198]	0.031 [a,c]	[0.02-0.098]	0.053 [c]	[0.014-0.238]	0.028 [a,c]	[0.021-0.062]	**0.0000**	**0.0000**
gondoic acid	0.184 [a]	[0.115-0.279]	0.348 [b]	[0.182-0.785]	0.180 [a]	[0.057-0.31]	0.254	[0.052-1.969]	0.193	[0.14-0.481]	**0.0010**	**0.0013**
linoleic acid	4.394	[1.382-14.933]	2.629	[0.472-6.298]	1.387	[0.915-5.789]	3.786	[0.584-45.629]	2.030	[0.81-22.663]	**0.0437**	**0.0483**
nervonic acid	0.174 [a]	[0.085-0.384]	0.906 [b]	[0.257-1.776]	0.199 [a]	[0.102-0.568]	0.270 [a]	[0.059-0.965]	0.189 [a]	[0.102-0.38]	**0.0000**	**0.0000**
oleic acid	4.084	[1.46-7.728]	3.129	[1.394-7.436]	2.169	[1.61-6.319]	4.862	[0.457-57.061]	2.555	[0.577-31.289]	0.2283	0.2283
palmitic acid	4.692	[2.026-8.149]	6.736	[4.477-16.048]	4.519	[1.704-11.44]	3.635	[0.891-30.053]	3.689	[2.043-14.354]	**0.0479**	0.0503
stearic acid	2.037 [a]	[1.195-4.366]	10.189 [b]	[2.838-18.753]	2.990	[0.785-13.644]	2.349 [a]	[0.433-23.969]	3.920	[0.95-10.71]	**0.0001**	**0.0001**
Sterols												
β-sitosterol	2.394 [a]	[1.354-4.42]	0.068 [b]	[0.039-0.746]	0.897 [b]	[0.385-2.815]	1.261 [c]	[0.415-5.989]	1.449 [a,c]	[0.975-1.887]	**0.0000**	**0.0000**
brassicasterol	0.032 [a]	[0.021-0.16]	0.020 [b]	[0.016-0.08]	0.032	[0.028-0.055]	0.049 [a]	[0.016-0.377]	0.098 [a]	[0.019-0.146]	**0.0001**	**0.0002**
campesterol	1.372 [a]	[0.739-2.422]	0.172 [b]	[0.062-0.685]	0.662 [a]	[0.313-1.529]	0.922 [a]	[0.31-2.672]	0.876 [a]	[0.432-1.553]	**0.0000**	**0.0000**
cholestanol	0.191 [a]	[0.071-0.473]	0.113 [b]	[0.047-0.232]	0.281 [a]	[0.152-0.798]	0.193 [a]	[0.032-0.746]	0.148	[0.125-0.48]	**0.0001**	**0.0002**
cholesterol	1.836 [a]	[1.182-5.67]	14.434 [b]	[2.283-22.874]	3.052 [a]	[1.522-11.444]	3.101 [a]	[0.648-14.617]	3.171 [a]	[0.877-6.193]	**0.0000**	**0.0000**
coprostanol	0.047 [a]	[0.022-0.607]	0.015 [b]	[0.01-0.643]	0.057 [a]	[0.023-5.464]	0.035 [a]	[0.011-1.494]	0.019	[0.013-0.221]	**0.0001**	**0.0002**
fucosterol	0.135 [a]	[0.077-0.233]	0.014 [b]	[0.011-0.056]	0.051 [a,c]	[0.022-0.075]	0.070 [c]	[0.028-0.672]	0.081 [a,c]	[0.056-0.667]	**0.0000**	**0.0000**
lathosterol	0.029 [a]	[0.02-0.068]	0.046 [b]	[0.026-0.109]	0.034	[0.025-0.096]	0.033	[0.021-0.108]	0.032	[0.022-0.066]	**0.0176**	**0.0205**
sitostanol	0.598 [a]	[0.207-1.253]	0.003 [b]	[0.002-0.055]	0.129 [a,c]	[0.058-0.242]	0.102 [c]	[0.014-1.236]	0.067 [b,c]	[0.031-0.109]	**0.0000**	**0.0000**
stigmasterol	0.483 [a]	[0.297-0.765]	0.034 [b]	[0.027-0.261]	0.176 [b]	[0.066-0.295]	0.429 [c]	[0.056-1.019]	0.668 [a,c]	[0.287-0.834]	**0.0000**	**0.0000**

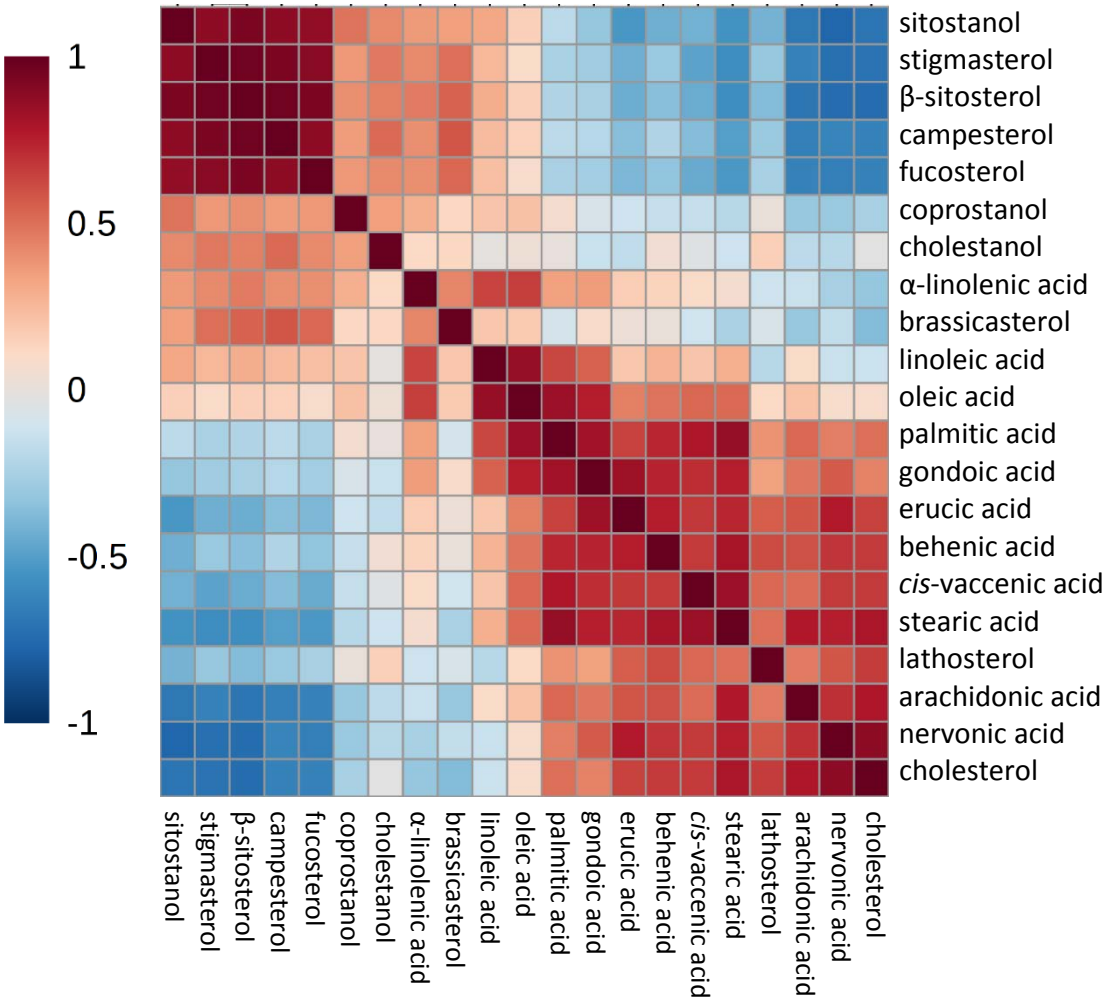


Figure 27. Pearson correlation matrix for baseline samples, including fecal samples from healthy control dogs and dogs with AHDS or IBD. The extent of correlation, by Pearson rho, is indicated by intensity of color from a strong negative correlation (*dark blue*) to a strong positive correlation (*dark red*).

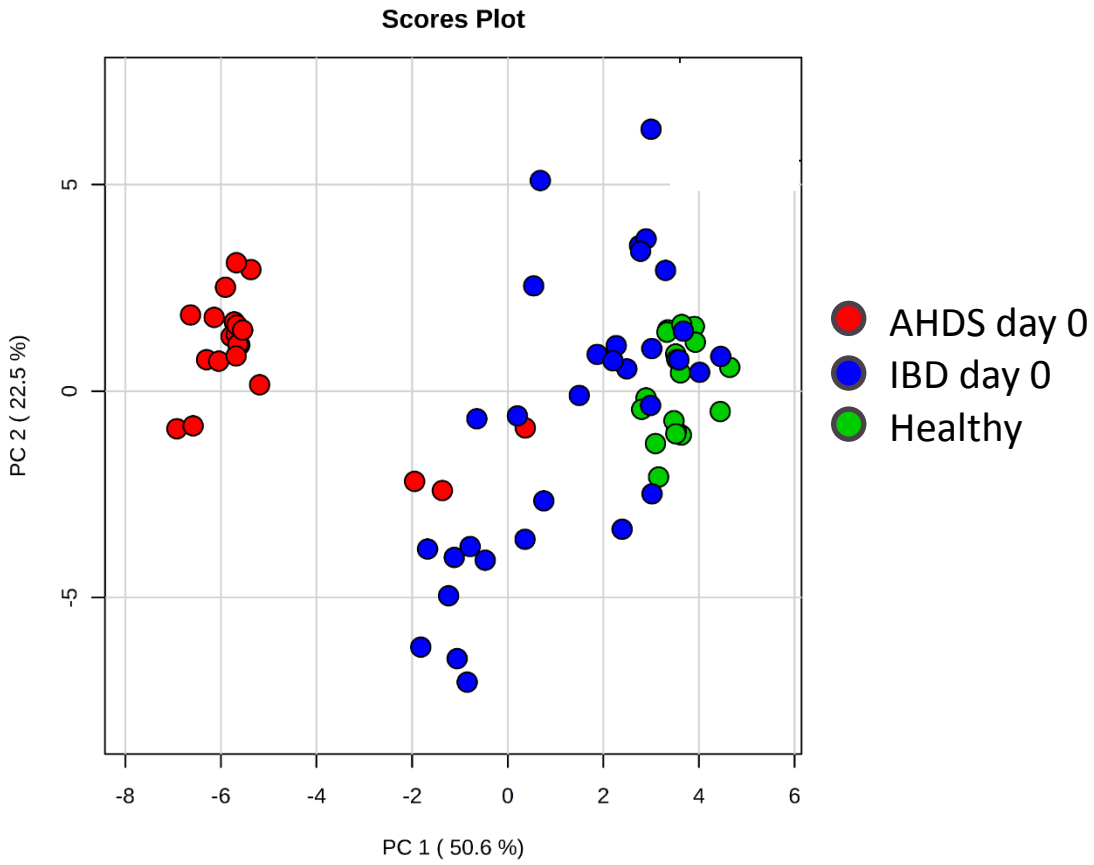


Figure 28. Principal components analysis (PCA) plot showing the similarity of fecal FASter profiles between dogs with AHDS, IBD, or healthy control dogs. Proximity of dots represents similarity of sample composition.

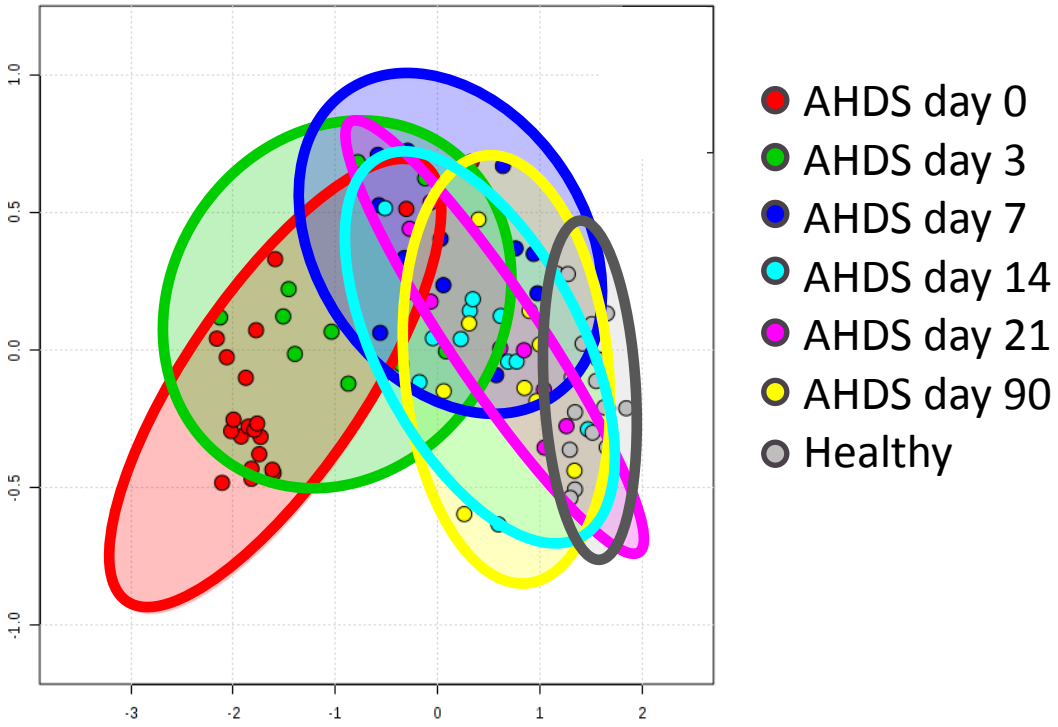


Figure 29. Principal components analysis (PCA) plot showing the progression of fecal FASter profiles of dogs with AHDS towards healthy control dogs. Proximity of dots represents similarity of sample composition. The shaded areas represent the 95% confidence area for the group.

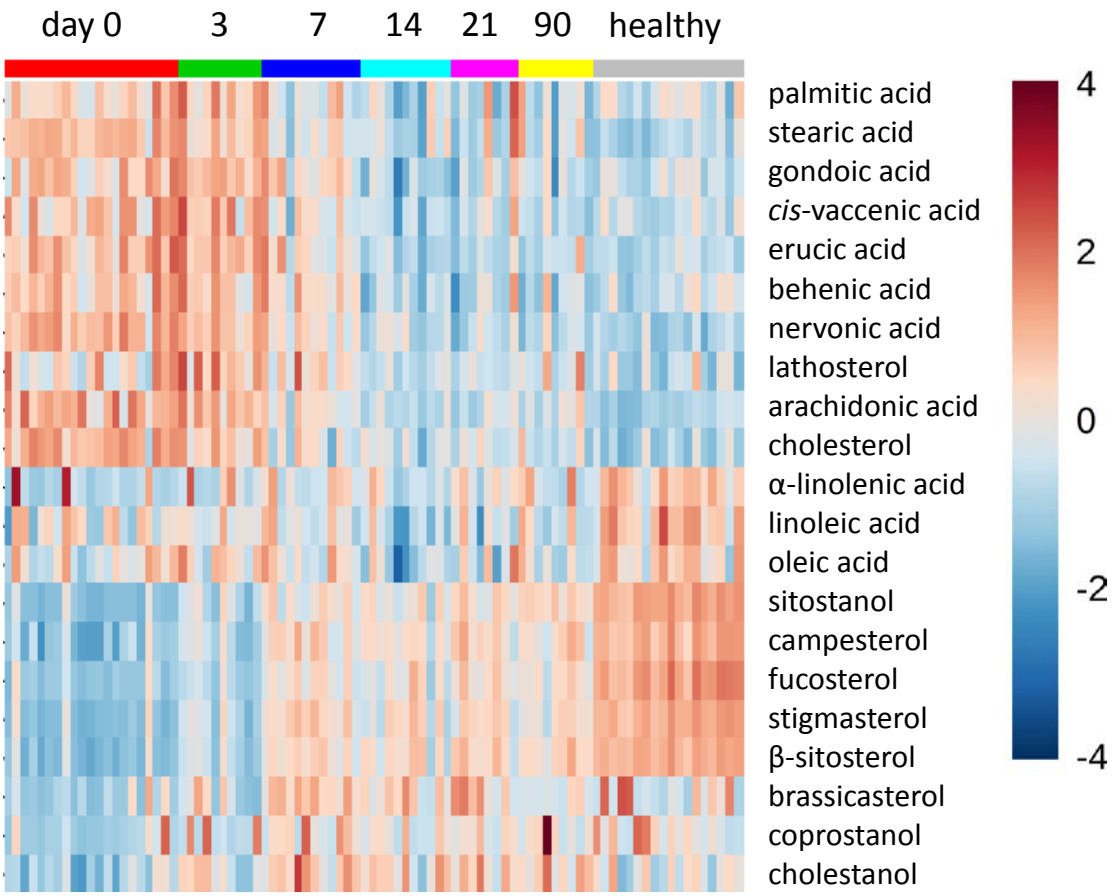


Figure 30. Heatmap showing distribution of metabolite concentrations across samples over time for the fecal FASter profiles of dogs with AHDS compared to healthy control dogs. The progression is most profound for the five phytosterols sitostanol, campesterol, fucosterol, stigmasterol, and β-sitosterol.

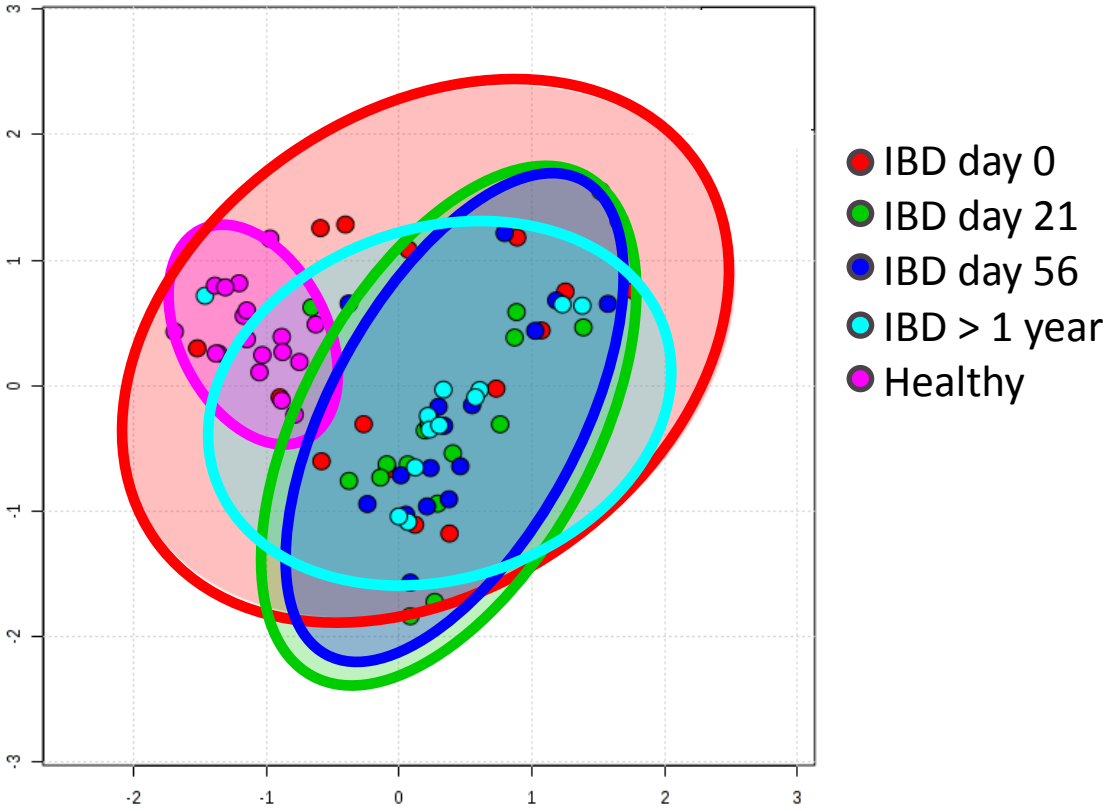


Figure 31. Principal components analysis (PCA) plot showing the progression of fecal FASter profiles of dogs with idiopathic IBD towards healthy control dogs. Proximity of dots represents similarity of sample composition. The shaded areas represent the 95% confidence area for the group.

8.3.2.3 Idiopathic IBD over time

The PCA plot showing the samples collected at each time point is shown in Figure 31. Samples are shown as dots; shaded areas represent the 95% confidence area for the group.

8.4 DISCUSSION

AHDS is an acute enteritis, while IBD is a chronic gastrointestinal disease. And although IBD still can be severe, the clinical presentation is usually not as dramatic as that of AHDS. Even more distinguishing between the two conditions is the response to treatment observed for these patients. With supportive therapy, most dogs with AHDS recover clinically within a few days. The data shown here, however, suggest that actual recovery may be incomplete even at 90 days after initial presentation, as the fecal FASter profiles approach but do not completely reach those seen in the healthy control dogs. This could in part be due to a confounding factor affecting the FASter profiles of the two groups, but noting the trajectory and progression of the AHDS samples even at day 21 to day 90, it is apparent that the compositions were continuing to shift towards a healthy profile. Also of note is that the shift was not directly linear along the principal components distinguishing healthy from day 0 of AHDS; rather, the group of samples deviated upwards and then back down as it trended to the right, towards the healthy population. This may translate physiologically to a nonlinear recovery process, which would not be surprising considering acute responses are often in contrast to long-term recovery. By looking at the heatmap for these samples, it subjectively appears that most persistent differences relate to lower concentrations on average of five phytosterols (sitostanol, campesterol, fucosterol, stigmasterol, and β-sitosterol). In the univariate analysis, stigmasterol and β-sitosterol were significantly altered between AHDS on day 90 and healthy dog samples.

The profiles over time from the dogs with IBD exhibited a markedly different progress compared to the dogs with AHDS, even though only dogs with IBD that clinically improved were included. While some shift and narrowing of the 95% confidence area is evident over time, it is remarkable that these profiles were nearly perfectly overlapping at day 21 and 56, and even after more than a year, the FASter profiles had barely changed, even though this group of dogs all

significantly improved clinically (data not shown). Importantly, a more global metabolomics view of these samples (unpublished data) reporting hundreds of metabolites did reflect more of a return towards health. The significance of the persistently altered FASter profiles may suggest either a permanent difference in the host metabolism (either genetics or adaptation due to disease or other medical history), or that clinical improvement does not necessarily translate into resolution at the biochemical level. More studies would be required to determine if the metabolites in the FASter profile may have utility in predicting relapse or long-term prognosis.

At baseline comparing the Pearson correlations of different metabolites, there is a strong positive correlation among five phytosterols (sitostanol, campesterol, fucosterol, stigmasterol, and β-sitosterol), and these have a strong negative correlation with arachidonic and nervonic acids, and with cholesterol. Although correlations are notoriously hazardous to draw conclusions from, observing them repeatedly is suggestive of a meaningful relationship. Speculating on the profound changes and rapid recovery observed among dogs with AHDS, one important feature that comes to mind is the extent of histological damage and subsequent bleeding into the GI tract. Because cell membranes (including red blood cells) are composed of large quantities of cholesterol and fatty acids to form the lipid bilayer, it would be reasonable to suspect this accounts for those changes observed. However, hematochezia typically resolves within a few days, so the altered FASter profiles persisting on days 7, 14, 21, and 90 cannot be explained by blood contamination. Another effect in the early stages may be decreased absorption of nutrients due to the major sloughing of mucosa, causing both an increase in non-absorbed material as well as adding sloughed cells and blood to the mix. This could again have a “diluting” effect on concentrations, but as before, this is not a likely explanation after the acute phase of the disease, though some persisting malabsorption is feasible.

Another possibility to consider, though there is no experimental evidence to either support or refute this explanation, is that gene expression mechanisms similar to those in play in IBD are also invoked in recovery from the acute onset of AHDS. The host must activate the immune system to protect itself while the mucosal barrier is impaired, and rebuilding the mucosal layer would create a tremendous demand for any available fatty acids and sterols, scavenging them from the lumen particularly in the small intestine where the surface area is particularly large. The disruption to the enterocytes, which are the gatekeepers of lipid absorption, may perpetuate the abnormal assimilation of lipids until the mucosa has had adequate time to recover structurally. Gene expression studies from biopsies in dogs recovering from AHDS would potentially help elucidate the role of host gene regulation during the recovery process.

Among dogs with IBD, dietary management is often ongoing. Though not shown in this study, unpublished work in our lab has suggested that diet can significantly alter these metabolite concentrations in healthy animals. However, taken subjectively from multivariate analyses, the observed shift in FASter profiles associated with feeding a commonly used hydrolyzed diet was not in the direction of the same principal components, meaning the effect of disease is likely distinct from the effect of dietary treatment. The significance of the altered metabolite concentrations induced by diet in the perpetuation of disease is an area that would require further studies.

8.5 CONCLUSIONS

The fecal FASter profile of dogs with AHDS was distinct from dogs with IBD and from healthy control dogs. The rapid recovery observed in AHDS patients clinically was recapitulated in the shifting composition of the fecal profiles towards the healthy group, yet was still abnormal after 90 days. Longer term follow up of dogs with AHDS would assist in determining if these

animals remain abnormal (similar to scarring) or if they eventually are associated with a complete restoration of a healthy fecal FASter profile.

The samples from dogs with IBD demonstrated persistence of an abnormal FASter profile, even after more than one year of treatment. The contribution of different diets (e.g., hydrolyzed, novel protein, or other types of diets) should be explored systematically to assess if some of these altered metabolite concentrations are iatrogenic, and how the features of a therapeutic diet should be considered.

9. FECAL CONCENTRATIONS OF FATTY ACIDS IN DOGS WITH EXOCRINE PANCREATIC INSUFFICIENCY RECEIVING ENZYME SUPPLEMENTATION

9.1 INTRODUCTION

Exocrine pancreatic insufficiency (EPI) is a disease characterized by insufficient synthesis and secretion of pancreatic enzymes by the exocrine pancreas, resulting in malassimilation of macro-nutrients. For example, insufficient pancreatic lipase prevents normal digestion of dietary fat. Consequently, EPI would be expected to be associated with excessive fat (e.g., fatty acids) remaining in the feces. Treatment of EPI includes oral supplementation with pancreatic digestive enzymes and is often effective at decreasing severity of clinical signs, but fat assimilation does not completely normalize. The aim of this study was to evaluate fecal fatty acid (FA) concentrations in dogs with EPI undergoing enzyme supplementation. The hypothesis of this study was that fecal fatty acid concentrations would be increased in dogs with EPI compared to those of healthy dogs, even when being successfully treated with enzyme supplementation.

9.2 MATERIALS AND METHODS

9.2.1 Study population and sample collection

Naturally-passed fecal samples from dogs with EPI (n=34) were retrospectively selected from surplus samples from other studies, stored at -80°C. In addition, there were samples from dogs with EPI who were not currently on enzyme supplementation (n=5) that were included only for visualizing the clustering of samples in a PCA plot. Samples from diseased dogs were categorized as EPI based on the diagnosis by the referring clinician in addition to a severely

decreased serum TLI concentration, and had to be on enzyme replacement therapy at the time of fecal collection. Stored samples from healthy dogs (n=72) from a variety of studies were used as the control group. Some control dogs were without a TLI result or other bloodwork, but all individuals were deemed healthy based on owner questionnaire and no reported clinical signs of gastrointestinal disease.

9.2.2 Sample preparation and instrument analysis

Samples were prepared and analyzed by the FASter assay using gas chromatography-mass spectrometry (GC-MS) as described in Section 6.2.

9.2.3 Statistical analyses

Results in μg/mg lyophilized feces for all samples were analyzed in JMP (SAS, Durham, NC, USA). Mann-Whitney *U* tests and a Benjamini-Hochberg step-up method to adjust for multiple comparisons were used to identify significantly altered compounds between dogs with EPI and healthy control dogs. Statistical significance was set at $p<0.05$. Multivariate analysis was performed using MetaboAnalyst (Xia et al. 2015; Xia and Wishart 2011, 2016). Raw concentrations were log-transformed and Pareto-scaled to achieve a more Gaussian distribution. Principal components analysis (PCA) plots were used to show the clustering/overlap among FASter profiles between dogs with EPI (with and without enzyme replacement therapy) and healthy control dogs. A plot of the Pearson correlation matrix was also prepared with all three groups included.

9.3 RESULTS

With the exception of nervonic acid, all fecal FAs were significantly increased in the feces of dogs with EPI. Among sterols, cholesterol was notably the same in both groups, while

coprostanol was decreased along with several phytosterols (i.e., sitosterol, campesterol, fucosterol, sitostanol, and stigmasterol). Data are summarized in Table 19.

The PCA plot showing the distribution of samples based on their similarity in terms of FASter profile composition is shown in Figure 32, and the correlation matrix plot is shown in Figure 33.

Table 19. Summary of fecal fatty acid and sterol concentrations in dogs with EPI compared to healthy control dogs.

	Healthy		**EPI**			
Compound	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
Fatty acids						
α-linolenic acid (18:3 n-3)	0.337	[0.075-3.479]	1.022	[0.202-5.503]	**0.0000**	**0.0000**
arachidonic acid (20:4 n-6)	0.413	[0.19-2.381]	0.745	[0.241-6.23]	**0.0000**	**0.0000**
behenic acid (22:0)	0.248	[0.097-0.519]	0.344	[0.147-1.105]	**0.0000**	**0.0000**
cis-vaccenic acid (18:1 n-7)	0.977	[0.47-4.851]	2.657	[0.225-9.477]	**0.0000**	**0.0000**
erucic acid (22:1 n-9)	0.034	[0.014-0.089]	0.074	[0.022-0.646]	**0.0000**	**0.0000**
gondoic acid (20:1 n-9)	0.190	[0.029-0.594]	0.690	[0.099-2.64]	**0.0000**	**0.0000**
linoleic acid (18:2 n-6)	4.075	[0.434-29.726]	10.343	[1.653-34.451]	**0.0000**	**0.0000**
nervonic acid (24:1 n-9)	0.182	[0.046-0.5]	0.187	[0.06-0.979]	0.1653	0.1928
oleic acid (18:1 n-9)	4.069	[0.321-16.733]	13.710	[1.78-68.848]	**0.0000**	**0.0000**
palmitic acid (16:0)	4.247	[1.283-13.422]	11.974	[1.57-48.4]	**0.0000**	**0.0000**
stearic acid (18:0)	2.277	[0.935-7.477]	6.623	[1.083-43.154]	**0.0000**	**0.0000**
Sterols						
β-sitosterol	2.108	[0.523-5.806]	1.240	[0.177-2.452]	**0.0000**	**0.0000**
brassicasterol	0.036	[0.017-0.48]	0.052	[0.015-0.38]	0.3554	0.3732
campesterol	1.269	[0.449-3.368]	0.793	[0.194-2.093]	**0.0000**	**0.0000**
cholestanol	0.210	[0.058-0.473]	0.102	[0.032-0.277]	**0.0000**	**0.0000**
cholesterol	1.928	[0.958-8.322]	2.055	[1.047-9.059]	0.8710	0.8710
coprostanol	0.049	[0.017-0.607]	0.033	[0.011-1.499]	**0.0024**	**0.0030**
fucosterol	0.117	[0.025-0.332]	0.066	[0.015-0.299]	**0.0000**	**0.0000**
lathosterol	0.033	[0.02-0.264]	0.029	[0.02-0.108]	0.3242	0.3584
sitostanol	0.461	[0.025-1.335]	0.042	[0.004-0.293]	**0.0000**	**0.0000**
stigmasterol	0.473	[0.122-1.142]	0.303	[0.075-0.804]	**0.0000**	**0.0000**

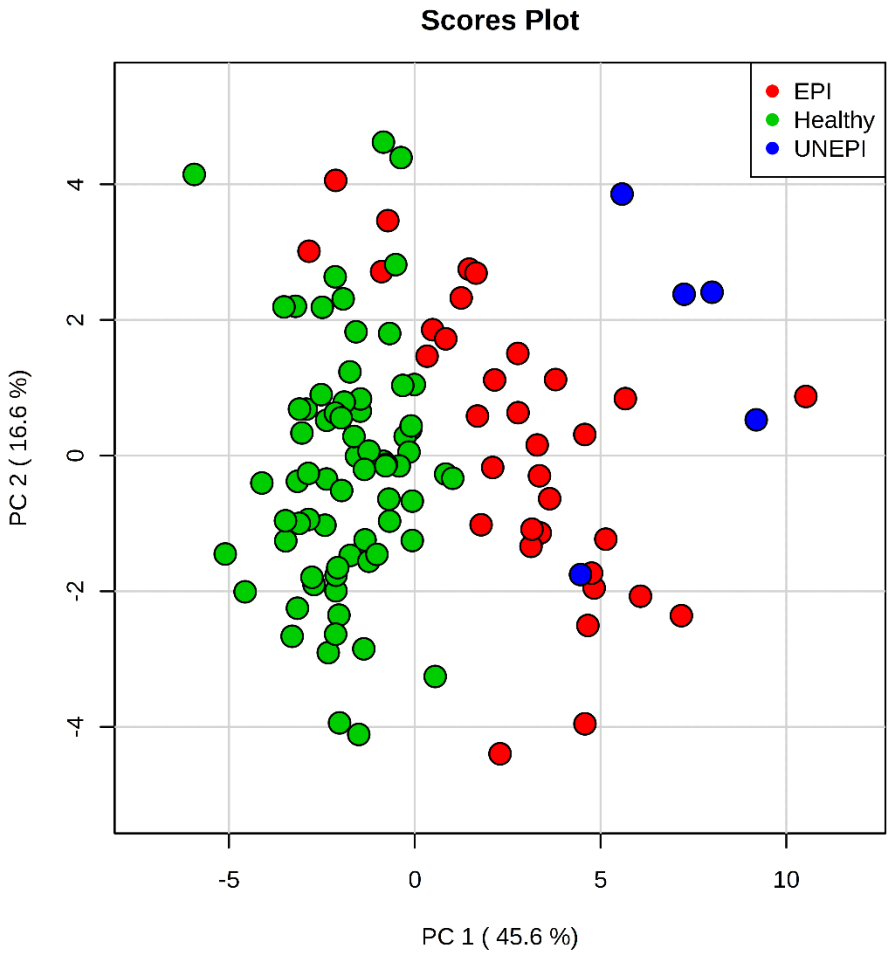


Figure 32. PCA plot showing fecal FASter profiles for healthy control dogs (*green*) compared to dogs with EPI undergoing enzyme replacement therapy (*red*) and untreated dogs with EPI (*blue*).

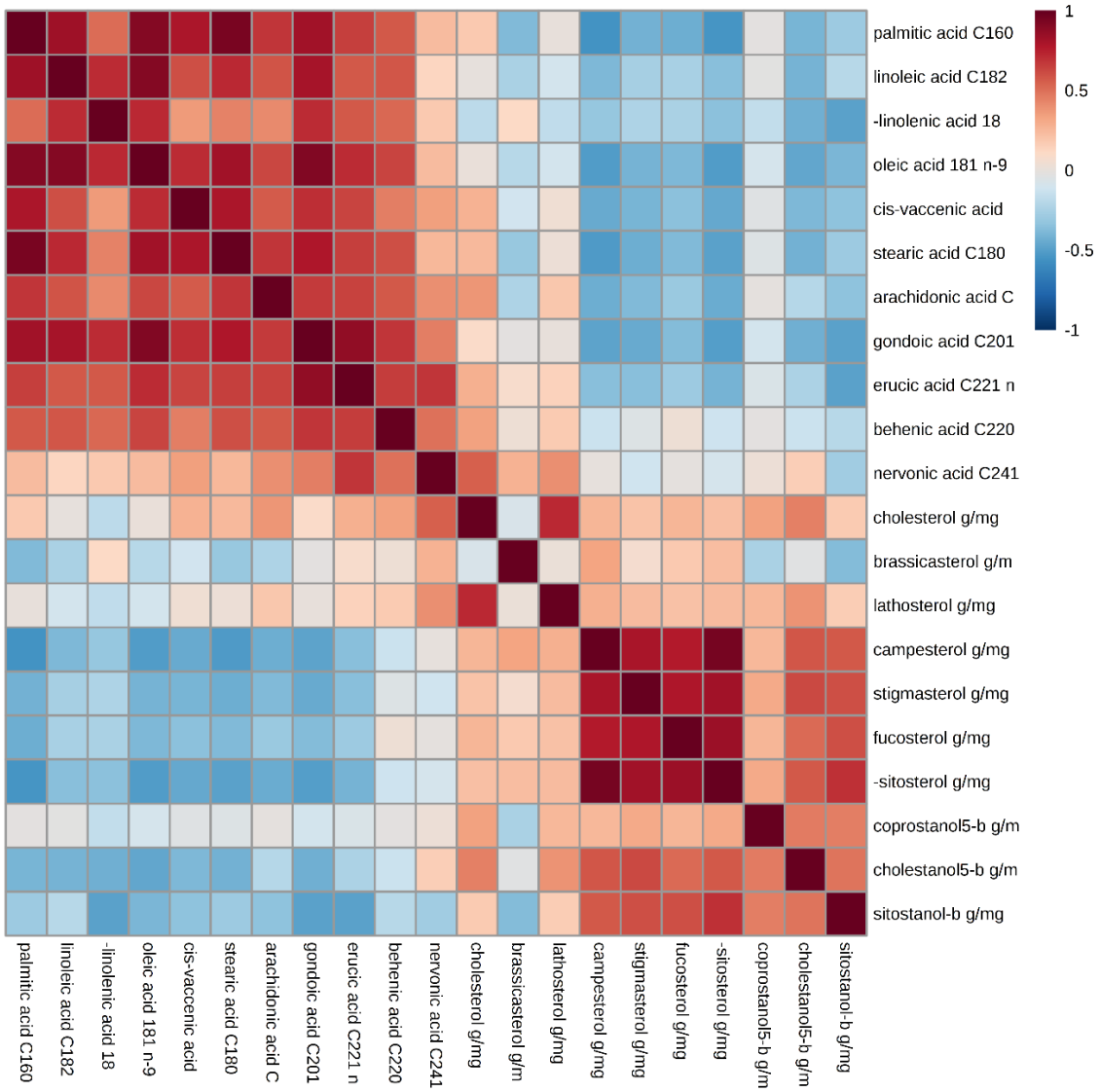


Figure 33. Pearson correlation matrix plot showing correlation between metabolites across the dogs with EPI and healthy control dogs.

9.4 DISCUSSION

With enzyme supplementation, the clinical signs of EPI can usually be well-controlled. Although the group of untreated dogs is very small, the PCA plot does suggest that most of them had more severe alterations to their fecal fatty acid and sterol composition than the dogs receiving enzyme replacement therapy. However, clearly most of the dogs on enzyme therapy remained abnormal based on the nearly-distinct clustering of dogs with EPI from the healthy control dogs. One feature that stands out from the data is the lack of difference in nervonic acid observed between healthy dogs and dogs with EPI, while every other fatty acid quantified was significantly increased in disease. The reason for this is unclear, but may in part be due to the length of nervonic acid (24 carbons; the longest fatty acid in the FASter panel). This is supported somewhat by the more positive correlation of nervonic acid to its biosynthetic precursor, erucic acid, and also to gondoic and behenic acids, the other VLCFAs. Metabolically, VLCFAs are degraded via peroxisomal β-oxidation before they can be transferred to mitochondria for complete oxidation, so perhaps the mechanisms controlling VLCFA absorption and metabolism are less affected than those for the more common dietary fatty acids. There may be a significant difference in dietary intake of some of these compounds, and a detailed dietary history, which would be required to control for this, was not available.

Also surprising is the lack of a difference in fecal cholesterol concentrations with concurrent alterations in phytosterols. This is quite distinct from the results of dogs with chronic enteropathy (e.g., IBD), where increased cholesterol concentrations were typically concurrent with decreased phytosterol concentrations. To determine whether this is related to contrasting mechanisms of disease certainly requires a better understanding of the underlying pathogenesis of CE. Importantly, our ability to understand altered fecal lipid concentrations is enhanced by observing the alterations occurring in a variety of different gastrointestinal diseases.

10. EFFECT OF CHOLESTYRAMINE ON FECAL CONCENTRATIONS OF STEROLS AND FATTY ACIDS IN HEALTHY DOGS

10.1 INTRODUCTION

Cholestyramine is a resin that forms insoluble complexes with bile acids, preventing intestinal reabsorption and enhancing fecal elimination of bile acids. By blocking enterohepatic circulation, feedback suppression of hepatic synthesis of bile acids is inhibited, thus increasing bile acid synthesis from cholesterol and decreasing serum cholesterol concentrations. While serum cholesterol is a well-studied parameter, recent work has also shown altered concentrations of fecal cholesterol and other sterols in dogs with gastrointestinal (GI) disease. Furthermore, GI assimilation of lipids is dependent on the formation of chylomicrons from triglycerides (i.e., glycerol and fatty acids) and esterified cholesterol. This study aimed to explore the effect of cholestyramine on lipid metabolism by assessing fecal concentrations of fatty acids and sterols in healthy dogs.

10.2 MATERIALS AND METHODS

A crossover study design was used. 12 healthy Beagle dogs from a research colony were randomized into groups A and B. Dogs in group A (n=6) received 11.4 g/day cholestyramine powder (8 g/day active ingredient) suspended in 75 mL of water for two weeks (period 1), a two week washout period, and nothing additional for two weeks (period 2). Dogs in group B (n=6) received treatments in reverse order. Fecal samples were collected two weeks prior to the start of period 1 and at the end of each treatment period. Samples were immediately frozen upon collection, and shipped as a batch on dry ice for analysis. Samples were prepared and analyzed by

the FASter assay using gas chromatography-mass spectrometry (GC-MS) as described in Section 6.2.

Effects were modeled by standard least squares (SLS) with a restricted maximum likelihood (REML) method in the statistical software package JMP (SAS, Durham, NC, USA). Carryover effect was assessed by interaction between drug and treatment period. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg step-up method and statistical significance was set at $p<0.05$. The changes in concentration were calculated as the median average change across individuals while on cholestyramine compared to the sample taken during the no-drug period. This net change value was compared to the median no-drug concentration to calculate the percent change.

10.3 RESULTS

None of the measured sterols or fatty acids exhibited a significant carryover effect. Concentrations of all measured sterols except lathosterol were significantly affected by cholestyramine. Interestingly, brassicasterol was significantly increased, while all other sterols quantified were decreased regardless of whether they were phytosterols (i.e., campesterol, stigmasterol, fucosterol, β-sitosterol, and sitostanol) or zoosterols (i.e., cholesterol, coprostanol, and cholestanol). Although α-linolenic acid was significantly decreased and linoleic acid was virtually unchanged, most fecal fatty acid concentrations were increased with cholestyramine (i.e., palmitic, oleic, *cis*-vaccenic, stearic, and arachidonic acids). However, the very long-chain fatty acids (VLCFAs; i.e., gondoic, erucic, behenic, and nervonic acids) were notably not significantly changed. Summary data are shown in Table 20.

Table 20. Summary data for fecal sterol and fatty acid concentrations as affected by cholestyramine administration.

Compound	End Cholestyramine Median (µg/mg)	End Cholestyramine Range (µg/mg)	End No Drug Median (µg/mg)	End No Drug Range (µg/mg)	Change (median)	Change (%)	*p*-value	*q*-value
Fatty acids								
palmitic acid	4.2159	[3.098-6.6523]	3.2664	[2.5144-4.3075]	0.6767	21%	**0.0163**	**0.0245**
linoleic acid	1.8812	[1.4178-3.3099]	1.9574	[1.7257-4.3757]	-0.2287	-12%	0.7340	0.7340
α-linolenic acid	0.2052	[0.162-0.3182]	0.2674	[0.1949-0.3961]	-0.0729	-27%	**0.0201**	**0.0282**
oleic acid	3.4869	[2.6718-5.7879]	2.9025	[2.4548-3.6656]	0.3038	10%	**0.0129**	**0.0210**
cis-vaccenic acid	2.1821	[0.8892-3.0069]	0.9132	[0.6164-1.5902]	1.2968	142%	**0.0012**	**0.0032**
stearic acid	3.2991	[2.3158-5.4361]	2.0602	[1.7659-3.1147]	0.9980	48%	**0.0130**	**0.0210**
arachidonic acid	1.4542	[0.8199-2.2534]	0.8847	[0.6337-1.2835]	0.5915	67%	**0.0099**	**0.0207**
gondoic acid	0.2726	[0.1474-0.3571]	0.1879	[0.1334-0.2997]	0.0594	32%	**0.0441**	0.0579
erucic acid	0.0428	[0.0291-0.0616]	0.0417	[0.0303-0.0499]	0.0043	10%	0.2054	0.2303
behenic acid	0.2609	[0.2241-0.4195]	0.2943	[0.2473-0.3873]	-0.0316	-11%	0.2084	0.2303
nervonic acid	0.2085	[0.1695-0.4613]	0.2423	[0.1741-0.3636]	-0.0321	-13%	0.3526	0.3702
Sterols								
cholesterol	1.9879	[1.3585-6.0684]	3.6176	[1.7063-8.7236]	-1.7386	-48%	**0.0122**	**0.0210**
brassicasterol	0.0486	[0.0333-0.0658]	0.0265	[0.0177-0.0357]	0.0215	81%	**0.0001**	**0.0007**
lathosterol	0.0327	[0.0226-0.1603]	0.0513	[0.0224-0.1739]	-0.0152	-30%	0.1571	0.1941
campesterol	0.4292	[0.3107-1.0009]	0.7491	[0.5391-1.0599]	-0.2152	-29%	**0.0007**	**0.0026**
stigmasterol	0.2375	[0.1896-0.375]	0.3223	[0.2712-0.3985]	-0.0577	-18%	**0.0009**	**0.0026**
fucosterol	0.0442	[0.034-0.0858]	0.0614	[0.0485-0.0792]	-0.0140	-23%	**0.0060**	**0.0141**
β-sitosterol	0.8146	[0.6833-1.594]	1.1775	[0.9233-1.5319]	-0.3295	-28%	**0.0008**	**0.0026**
coprostanol(5β)	0.0510	[0.0392-0.0758]	0.0898	[0.0613-0.1588]	-0.0258	-29%	**0.0003**	**0.0017**
cholestanol(5α)	0.1338	[0.102-0.3307]	0.2904	[0.2085-0.5027]	-0.1500	-52%	**0.0003**	**0.0017**
sitostanol	0.1461	[0.1066-0.19]	0.2009	[0.1696-0.2467]	-0.0601	-30%	**0.0000**	**0.0001**

10.4 DISCUSSION

The administration of cholestyramine resulted in altered concentrations of fecal sterols and fatty acids. The physiologic interrelationship of these compounds in lipid metabolism suggests that cholestyramine may have a significant effect on intra-luminal homeostasis of sterols and assimilation of fatty acids. The mechanism of action of cholestyramine is known to be increased fecal excretion of bile acids. This would be expected to diminish the feedback inhibition on the FXR nuclear receptor, inciting a cascade of changes in lipid metabolism pathways as cholesterol

from the body is drawn upon and converted to bile acids. Although all cells possess the ability to synthesize cholesterol *de novo*, a very large portion of cholesterol is thought to originate from the diet, so increased GI absorption of cholesterol would be logical for an immediate response, most likely modulated by NPC1L1 receptors that do not exclude phytosterols. Simultaneously, the decreased availability of bile acids would impair the formation of lipid micelles during digestion, thus decreasing the ability of the enterocytes to absorb luminal fatty acids and resulting in a net increase in fecal concentrations of fatty acids. It is also thought that cholestyramine in the lumen can directly bind fatty acids to an extent, which also could increase luminal concentrations of fatty acids. Yet some selectivity appears to occur in this regard, causing particularly extreme increases in *cis*-vaccenic acid and arachidonic acid, and a decrease in α-linolenic acid. As shown in Section 3, among the fatty acids, *cis*-vaccenic and arachidonic acids are particularly large components of bile compared to their concentrations in feces. As the body responds to try to restore bile acid homeostasis, it would make sense that the gall bladder contents are more completely expelled. However, as arachidonic acid has repeatedly been shown to be related to inflammation, the increase in concentration may indicate a detrimental effect overall. The only omega-3 fatty acid quantified, α-linolenic acid, was somewhat depleted from the lumen. Since the ratio of omega-6 to omega-3 fatty acids is thought to be the relevant parameter for pro-inflammatory relationships, the physiological significance of opposing changes between arachidonic and α-linolenic acids may be particularly profound. The lack of changes among other VLCFAs is also appreciable. This may suggest that the regulation of VLCFAs is via distinct mechanisms from LCFAs.

Although serum cholesterol measurements were not obtained, these animals were not hypercholesterolemic so it is unclear whether serum cholesterol would have decreased with cholestyramine treatment. By inducing a whole-body depletion of cholesterol, it would make sense that mechanisms of sterol absorption would be upregulated after two weeks of cholestyramine

administration. Since targeted metabolomics studies have shown detrimental effects associated with decreased concentrations of fecal phytosterols, and anti-inflammatory effects associated with oral phytosterols administration in mouse models of colitis (Aldini et al. 2014), it should be considered whether this may be a negative side effect of cholestyramine administration.

Future studies are required to explore the effect of cholestyramine on fecal metabolite concentrations in dogs with GI disease.

11. CONCLUSIONS

The work reported in this dissertation represents a very large body of data, much of which is descriptive, but provides a stepping-stone for future research projects. First, the utility of feces as a biological specimen has been demonstrated. Out of all possible sample types, collection of naturally passed feces is among the least invasive and least expensive of any (on par with a free-catch urine sample), easily carried out by the pet owner without a visit to the veterinarian, and sample quantity is usually abundant. Even with the variety of dietary intake across individuals, the net effects of the gastrointestinal microbiota and host metabolism in disease states clearly exerts an effect on the biochemical composition of feces. Although future work should attempt to include a dietary history as initial data from our laboratory have shown that dietary changes result in a different shift than what is observed in disease states. An interesting study to improve our understanding of how different diets affect the fecal composition would be to perform metabolomics studies on paired samples of diet (e.g., the commercial kibble) and feces.

The breadth of biochemical composition within the GI tract was demonstrated by analyzing the metabolome at different sites of the canine GI tract. Metabolomics has historically been used predominantly to study serum samples, but host physiology typically keeps tight control over the circulatory system. While that makes serum a quieter matrix, it also likely eliminates many of the effects that are occurring at the level of the host-microbiota interaction within the GI tract.

As feces have become more appreciated as a sample for molecular analysis, the question arises regarding the use of feces as a surrogate for other locations within the GI tract. In this study, we used both untargeted and targeted metabolomics to compare the duodenal, ileal, colonic, and rectal contents in healthy dogs. The results provide a context for interpretation of other studies by

elucidating the changing concentrations of metabolites and also the composition of the microbiota in different intestinal segments. However, the use of fecal samples for analysis is also associated with unique challenges. For example, the total amount of food taken in orally versus the relatively small amount of feces released upon defecation, and the concentrating effect that occurs towards non-absorbed compounds due to absorption of nutrients. Similar to the use of creatinine or specific gravity to normalize for water content of urine, perhaps there is a way to normalize for percent absorption of digesta, but this has not been described as of yet. Ultimately, one can also argue that the relative concentrations of compounds are still fundamentally the important feature, especially when comparing samples from diseased dogs to those from healthy dogs from the same site (e.g., duodenal chyme, feces, or other), and that feces represent the net effect of all factors.

A particular area of interest in veterinary research is canine chronic enteropathy. Using untargeted metabolomics to compare the fecal metabolome of dogs with CE to healthy control dogs revealed a tremendously diverse group of chemical alterations. Furthermore, by grouping the compounds into pathways based on what is known about metabolism and biochemical pathways, certain differences in metabolism are of interest. Most notably, lipid metabolism as evidenced by altered abundances of sterols and fatty acids. However, many other metabolic pathways appeared to be disturbed, including aromatic amino acid metabolism, redox, and most excitingly, pathways involving host-microbiota interactions and interdependence. A more complete understanding of these interactions is required to completely appreciate how the microbiota and metabolome can be leveraged for diagnostic, prognostic, and therapeutic benefits, but the potential is immense, especially considering the vast and malleable metabolic repertoire of the microbiota.

Regarding lipid metabolism, there is a significant body of research exploring connections between regulation of host gene expression as well as involvement of the microbiota. Specific examples include the conversion of cholesterol to coprostanol and the association between this

ability and decreased risks for certain gastrointestinal diseases, and also the relationships between diseases and the bile acid pool (where the microbiota are credited with dehydroxylating primary bile acids to form secondary bile acids). Data from an extensive gene expression study on canine duodenal mucosal biopsies provided many suggestions of altered host gene expression related to lipid metabolism, including neurotensin, fatty acid binding proteins, fatty acid transport proteins, several members of acyl-CoA synthetases, fatty acid elongases, PPARγ, FXR, and SREBF2, among many others known to play a role in fatty acid, bile acid, and sterol homeostasis. By combining the data previously published with our work regarding the fecal metabolome, it is quite clear that a significant dysmetabolism of lipids occurs in the gastrointestinal diseases studied.

A quantitative in-house assay was developed as part of this study, to allow direct comparison of samples with regard to their fatty acids and sterols concentrations. As this assay was applied to multiple cohorts of dogs with chronic enteropathy, certain findings were consistent between various diseases: decreased concentrations of phytosterols and increased cholesterol in the feces of dogs with chronic enteropathy. The study of fecal sterols in the context of IBD is quite novel, though there is some research regarding non-cholesterol sterols in serum. We have not yet proposed a molecular mechanism for these alterations, but there is ample room for applying techniques such as immunohistochemistry to expand upon the initial data regarding gene expression. Perhaps even more practical would be to expand the breadth of fatty acids included in the assay. Among the notable changes reported in the literature, humans with IBD are thought to deplete their *n*-3 fatty acids, which may perpetuate inflammation by changing the *n*-6 to *n*-3 ratio. The only *n*-3 fatty acid that is currently part of our assay is arachidonic acid, and although it is undeniably a key player in inflammation, including other polyunsaturated fatty acids would likely increase the yield of information gained from each sample. Another group of compounds that appears to be relevant but is not currently being analyzed is the group of dicarboxylic acids. These

were observed to be significantly decreased in abundance in feces of dogs with CE in an untargeted metabolomics study, suggesting potential changes in ω-oxidation of fatty acids. Among the more critical parts of assay development remaining is the determination of a reference interval for each analyte. Very stringent criteria to identify a small group of healthy control animals resulted in similar variability as loose criteria to obtain a large group of healthy animals, suggesting that the significant findings persist across the broader population. However, the purpose of the assay for diagnostic testing does require establishing parameters for classification of animals with clinical signs suggestive of gastrointestinal disease.

This work has shown that different disease phenotypes are accompanied by different fecal fatty acid and sterol profiles. The changes in diseased animals relative to healthy animals are summarized in Table 21, along with the changes observed during cholestyramine administration. Since very few changes of the phytosterols were compound-specific, they were considered as a group for the purposes of assessing trends across conditions.

Comparing acute and chronic gastrointestinal diseases, there was no perfect correlation between clinical recovery and FASter profiles, but it was clear that the fecal composition in dogs with acute hemorrhagic diarrhea syndrome progressed over time towards a profile resembling the healthy control animals. Of note, the pre-disease FASter profiles are unknown, so there is some chance that these animals have recovered to their pre-disease state, and the profiles would indicate a predisposition to disease. Continuing to follow animals after recovery from AHDS over a longer duration may reveal either ongoing recovery, or potentially prolonged "scarring" to their metabolic pathways that may affect their predisposition to other diseases. Conversely, the animals with chronic GI disease, specifically idiopathic IBD, exhibited very little change in fecal FASter composition over time when examined from a multivariate perspective. Most studies in canine IBD have been limited to shorter follow up periods, so the progression/remission of disease over

a longer period and in a more diverse group of patients would help elucidate more subtle nuances within these patterns.

Fecal samples from dogs with EPI were also analyzed, showing that in spite of enzyme replacement therapy, their fecal fatty acids and sterols profiles still showed an abnormal composition. The fact that cholesterol was not altered in this group, however, highlights that there is a different pattern of alteration among dogs with EPI than among dogs with IBD. This suggests that the FASter assay has the potential to distinguish diseases and elucidate aspects of pathogenesis by considering which components of lipid metabolism have been affected.

Table 21. Summary of changes in FASter assay compounds relative to healthy animals. Significantly increased concentrations are noted with a "+", decreases with a "–", and no significant change with a "0".

Condition / **Compound**	EPI	AHDS (day 0)	IBD	cholestyramine
[phytosterols]	–	–	–	–
cholesterol	0	+	+	–
nervonate (24:1 *n*-9)	0	+	0	0
behenate (22:0)	+	+	0	0
erucate (22:1 *n*-9)	+	+	+	0
gondoate (20:1 *n*-9)	+	+	0	0
arachidonate (20:4 *n*-6)	+	+	+	+
cis-vaccenate (18:1 *n*-7)	+	+	0	+
α-linolenate (18:3 *n*-3)	+	–	0	–
linoleate (18:2 *n*-6)	+	–	0	0
oleate (18:1 *n*-9)	+	0	0	+
stearate (18:0)	+	+	0	+
palmitate (16:0)	+	0	0	+

An additional study in our lab sought to perturb cholesterol homeostasis in healthy dogs by administering cholestyramine for a period, then quantifying the fecal FASter composition. The mechanism of action of cholestyramine is known to be increased fecal excretion of bile acids, and the alterations to lipid metabolism likely begin with diminished feedback inhibition of the FXR nuclear receptor. It is of great interest that inducing a dysmetabolism via bile acid depletion (and subsequent cholesterol depletion) resulted in a different pattern than the other diseases studied, again showing that the FASter assay has the potential to be used to assess different underlying etiologies on the basis of different patterns.

To speculate about the physiology underlying each disease pattern, first we must disregard the confounding factor of fecal mass: in other words, we assume that a lower concentration of a specific analyte corresponds to an absolute decrease in the amount exiting the body, rather than a dilution of the concentration by decreased absorption of non-analyte material. Since we know EPI, AHDS, and IBD are all characterized by some loss of function, this is probably a poor assumption to make. Nonetheless, this does not preclude speculation regarding compounds that are increased in concentration, and where the magnitude of the decrease is particularly profound, also speculating that the decreased concentrations are genuine indicators of alterations in the physiology. With regard to phytosterols, it appears they exhibit decreased concentrations in EPI, AHDS, and IBD, as well as upon administration of cholestyramine. The exception of brassicasterol being increased in feces of dogs administered cholestyramine could be related to the composition of the cholestyramine powder, but this has not explicitly been tested. In general, direct interference of cholestyramine with the quantification of fecal analytes should be assessed to determine if the resin masks the presence of certain compounds. Further analysis into specific ratios within the phytosterols (i.e., investigating relative biohydrogenation of β-sitosterol and stigmasterol to sitostanol) is ongoing and initial results suggest the presence of more nuanced patterns within the

phytosterols, and obviating the concern that dilution via decreased general absorption could be misleading. Fecal cholesterol was more variable, decreasing upon administration of cholestyramine, but unchanged in dogs with EPI and increased in dogs with AHDS or IBD. The variation among diseases was most profound looking at the concentrations of fatty acids. Nervonic acid, the longest chain fatty acid quantified in the panel, was unchanged from healthy control dogs except in dogs with AHDS. Nervonic acid is an important component of many cerebrosides and sphingomyelins, which are part of the lipid content of myelin. The exact mechanism of the extravasation of fluid into the GI tract in dogs with AHDS is unknown, but perhaps the sloughed mucosa combined with fluid movement from the cardiovascular space through the submucosa and submucosal plexus can carry and expose an appreciable amount of myelin breakdown products to the lumen. Another feature of samples from dogs with AHDS was the magnitude of increased cholesterol concentration. As was mentioned, cell membranes are composed largely of phospholipids and cholesterol, so this increase in the fecal material may be due to increased sloughing of enterocytes or red blood cells, or potentially accompanying the fluid as plasma cholesterol or myelin.

The hallmark of EPI using the FASter assay was increased fecal fatty acid concentrations, excepting only nervonic acid. Oleic, stearic, and palmitic acids are likely the largest contributors of dietary intake of fatty acids, and all three were increased in feces from dogs with EPI. This fits well with the understanding of the pathogenesis of the disease, primarily characterized by insufficient enzymes to digest and assimilate dietary fat (and other nutrients), thus leading to more of these macronutrients remaining in the feces. Supplementation of the diet of dogs with EPI with fish oil, a source of omega-3 fatty acids, is sometimes recommended along with vitamin E supplementation, and the direct effect of these compounds on the fecal concentrations of fatty acids in healthy dogs has not yet been determined.

The other group of dogs that also demonstrated increased C16 and C18 fatty acids was the group receiving cholestyramine. In this case, there is a known perturbation to lipid metabolism instigated by bile acid depletion in otherwise metabolically-normal animals. This may be explained by the ongoing demand for bile acids in the GI tract, which promotes secretion of bile into the duodenum. The C16 and C18 fatty acids (especially *cis*-vaccenic acid) are particularly concentrated in bile, so this would be expected to increase fecal concentrations of these compounds.

Other VLCFAs (behenic, erucic, and gondoic acids) were unchanged in dogs after cholestyramine administration, but increased in dogs with EPI or AHDS, and moderately trended upwards in dogs with IBD. Since peroxisomal oxidation is required to process VLCFAs before they can be used as substrate by the mitochondria, the accumulation in dogs with EPI, AHDS, or IBD may indicate either a defect in peroxisomal oxidation or demand on the pathway that exceeds the enzymatic turnover capacity. The observation in the gene expression study (Wilke et al. 2012) of 4-fold increased PPARγ supports that this pathway is affected in IBD, but the complexity of functions associated with that receptor prevents a sensible interpretation of the underlying mechanism. It may be as simple as upregulation due to increased ligand availability, or as complex as part of an underlying genetic defect that predisposes animals to a cascade of pro-inflammatory events. In contrast, arachidonic acid appears to increase with cholestyramine administration as well as with EPI, AHDS, or IBD compared to healthy control dogs. Arachidonic acid holds a critical role in the balance between pro- and anti-inflammatory mediators. It is the precursor to prostaglandins, leukotrienes, and thromboxanes, with the proportioning of synthesis of these metabolites depending on regulation of many distinct enzymes. It can be synthesized from linoleic acid, or can be obtained through dietary sources. It might be presumed that increased arachidonic acid acts as a perpetuator of inflammation: if the metabolic pathways leading to pro-inflammatory

products are upregulated, increased substrate may perpetuate the inflammation. However, dietary arachidonic acid itself does not seem to induce a pro-inflammatory state, though it may inhibit the anti-inflammatory effects of omega-3 fatty acids. This may explain the decreased fecal concentration of the omega-3 fatty acid α-linolenate observed with cholestyramine administration or in dogs with AHDS as the pro-inflammatory pathways would not be chronically upregulated and homeostasis of the inflammatory balance may increase the absorption of omega-3 fatty acids from the intestinal lumen compared to healthy animals. Furthermore, arachidonic acid is concentrated in bile, and as discussed above, the increased bile secretion induced by luminal bile acid depletion with cholestyramine administration would increase luminal arachidonic acid concentration without necessarily inducing a pro-inflammatory effect.

The pathogenesis of IBD is poorly understood. The results from the untargeted metabolomics study described in Section 5 suggest far more extensive changes to lipid metabolism than are evidenced by looking only at the compounds in the FASter assay, but results from all studies taken together strongly suggest that peroxisomal oxidation of fatty acids plays an important role in the dysmetabolism, whether causing or induced by changes in the role of the peroxisome in redox homeostasis concurrently.

A final simplified summary regarding the most notable alterations in compounds from the FASter assay and a corresponding speculation of the explanation is shown in Table 22. However, this study has raised far more questions than it has answered, and ultimately shows profound patterns of altered metabolism evidenced in fecal samples. Systematic evaluations of the effect of diet, supplements, antibiotics and other medications, inflammation, and the relationships with the microbiota are warranted and underway.

Table 22. Examples of the most notable changes observed in compounds of the FASter assay, comparing "condition" to healthy, and potential physiological explanations.

condition	notable fecal FASter concentration changes	potential explanation
AHDS	increased nervonate, increased cholesterol	cell membrane material from sloughed enterocytes or red blood cells; extravasation of lipid-rich fluid through the submucosa carrying plasma cholesterol solubilizes nervonic acid-enriched lipids from the submucosal plexus
EPI	all fatty acids increased (except nervonate)	lack of pancreatic lipase results in a significant increase in undigested and/or unassimilated fats remaining in the intestinal lumen
IBD	increased arachidonic acid, increased cholesterol	underlying aberration of fatty acid metabolism induces a chronic pro-inflammatory state, perpetuated by arachidonic acid; ongoing cell sloughing and lymphatic fluid high in cholesterol leaking into the intestinal lumen
cholestyramine	decreased cholesterol, variable fatty acid changes	physiological response to lack of luminal bile acids promotes secretion of bile; fatty acids that were increased are components of bile; cellular α-linolenic acid demand is increased to maintain homeostasis with presence of increased arachidonic acid

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APPENDIX

Table A-1. Significantly altered community functional potential as categorized by KEGG pathways.

L1			L2			L3		
Pathway Category	***p*-value**	***q*-value**	**Pathway Category**	***p*-value**	***q*-value**	**Pathway Category**	***p*-value**	***q*-value**
Cellular Processes	0.002	**0.015**	Cell Growth and Death	0.267	0.549	Meiosis - yeast	0.006	**0.029**
						p53 signaling pathway	0.005	**0.023**
			Cell Motility	0.002	**0.027**	Bacterial motility proteins	0.000	**0.013**
						Cytoskeleton proteins	0.001	**0.013**
						Flagellar assembly	0.001	**0.013**
			Transport and Catabolism	0.051	0.209	Lysosome	0.001	**0.016**
						Peroxisome	0.008	**0.034**
Env. Info. Processing	0.641	0.855	Membrane Transport	0.588	0.779	Bacterial secretion system	0.011	**0.043**
						Secretion system	0.001	**0.013**
			Signal Transduction	0.003	**0.027**	Two-component system	0.002	**0.020**
Genetic Info. Processing	0.416	0.686	Folding, Sorting and Degradation	0.253	0.549	Protein processing in endoplasmic reticulum	0.003	**0.023**
						Sulfur relay system	0.008	**0.034**
						Ubiquitin system	0.002	**0.020**
			Transcription	0.383	0.655	Basal transcription factors	0.004	**0.023**
			Translation	0.268	0.549	RNA transport	0.004	**0.023**
Human Diseases	0.004	**0.015**	Cancers	0.141	0.387	Bladder cancer	0.005	**0.025**
						Colorectal cancer	0.005	**0.023**
						Renal cell carcinoma	0.003	**0.023**
						Small cell lung cancer	0.005	**0.023**
			Cardiovascular Diseases	0.005	**0.027**	Viral myocarditis	0.005	**0.023**
			Infectious Diseases	0.004	**0.027**	African trypanosomiasis	0.002	**0.020**
						Bacterial invasion of epithelial cells	0.010	**0.040**
						Chagas disease (American trypanosomiasis)	0.002	**0.019**
						Influenza A	0.005	**0.023**
						Pertussis	0.013	**0.049**
						Shigellosis	0.010	**0.041**
						Toxoplasmosis	0.005	**0.023**
						Vibrio cholerae pathogenic cycle	0.005	**0.025**
			Neurodegenerative Diseases	0.003	**0.027**	Amyotrophic lateral sclerosis (ALS)	0.000	**0.013**
						Huntington's disease	0.003	**0.020**
						Parkinson's disease	0.004	**0.023**
Metabolism	0.851	0.936	Amino Acid Metabolism	0.832	0.898	Lysine degradation	0.001	**0.013**
						Tryptophan metabolism	0.000	**0.013**
						Valine, leucine and isoleucine degradation	0.002	**0.020**
			Biosynthesis of Other Secondary Metabolites	0.082	0.279	Caffeine metabolism	0.012	**0.049**
						Phenylpropanoid biosynthesis	0.000	**0.013**
						Streptomycin biosynthesis	0.005	**0.023**
			Carbohydrate Metabolism	0.761	0.865	Ascorbate and aldarate metabolism	0.004	**0.023**
			Glycan Biosynthesis and Metabolism	0.036	0.163	Glycosaminoglycan degradation	0.002	**0.019**
						Glycosphingolipid biosynthesis - globo series	0.001	**0.013**
						Glycosyltransferases	0.007	**0.029**

L1			L2			L3		
Pathway Category	***p*-value**	***q*-value**	**Pathway Category**	***p*-value**	***q*-value**	**Pathway Category**	***p*-value**	***q*-value**
						Lipopolysaccharide biosynthesis	0.004	**0.023**
						Lipopolysaccharide biosynthesis proteins	0.004	**0.023**
						Other glycan degradation	0.001	**0.013**
			Lipid Metabolism	0.577	0.779	alpha-Linolenic acid metabolism	0.001	**0.013**
						Biosynthesis of unsaturated fatty acids	0.001	**0.015**
						Fatty acid metabolism	0.012	**0.049**
						Linoleic acid metabolism	0.001	**0.013**
						Primary bile acid biosynthesis	0.000	**0.013**
						Secondary bile acid biosynthesis	0.000	**0.013**
						Sphingolipid metabolism	0.001	**0.013**
						Steroid biosynthesis	0.004	**0.023**
			Metabolism of Cofactors and Vitamins	0.740	0.865	Lipoic acid metabolism	0.003	**0.023**
						Ubiquinone and other terpenoid-quinone biosynthesis	0.002	**0.019**
			Metabolism of Other Amino Acids	0.311	0.606	beta-Alanine metabolism	0.005	**0.025**
						Cyanoamino acid metabolism	0.003	**0.020**
						Glutathione metabolism	0.001	**0.017**
			Metabolism of Terpenoids and Polyketides	0.596	0.779	Biosynthesis of siderophore group nonribosomal peptides	0.003	**0.023**
						Biosynthesis of vancomycin group antibiotics	0.002	**0.019**
						Geraniol degradation	0.001	**0.013**
						Limonene and pinene degradation	0.004	**0.023**
						Polyketide sugar unit biosynthesis	0.001	**0.013**
			Xenobiotics Biodegradation and Metabolism	0.466	0.764	Atrazine degradation	0.008	**0.034**
						Caprolactam degradation	0.003	**0.020**
						Chlorocyclohexane and chlorobenzene degradation	0.004	**0.023**
						Drug metabolism - cytochrome P450	0.005	**0.025**
						Fluorobenzoate degradation	0.001	**0.013**
						Metabolism of xenobiotics by cytochrome P450	0.001	**0.016**
						Toluene degradation	0.002	**0.020**
Organismal Systems	0.243	0.649	Circulatory System	0.004	**0.027**	Cardiac muscle contraction	0.004	**0.023**
			Endocrine System	0.093	0.292	Melanogenesis	0.013	**0.049**
			Environmental Adaptation	0.528	0.779	Circadian rhythm - plant	0.005	**0.025**
			Excretory System	0.002	**0.027**	Proximal tubule bicarbonate reclamation	0.002	**0.020**
Unclassified	0.429	0.686	Cellular Processes and Signaling	0.326	0.608	Cell motility and secretion	0.002	**0.020**
						Electron transfer carriers	0.001	**0.013**
						Germination	0.001	**0.017**
						Inorganic ion transport and metabolism	0.001	**0.013**
						Membrane and intracellular structural molecules	0.005	**0.023**
						Pores ion channels	0.003	**0.022**
						Sporulation	0.006	**0.026**
			Genetic Information Processing	0.685	0.851	Restriction enzyme	0.012	**0.049**
						Transcription related proteins	0.005	**0.025**
			Metabolism	0.351	0.626	Glycan biosynthesis and metabolism	0.002	**0.019**
						Metabolism of cofactors and vitamins	0.000	**0.013**
						Nucleotide metabolism	0.005	**0.025**

Table A-2. Medians and ranges of normalized peak areas for all detected named metabolites. Compounds identified only by comparison to published library spectra are marked with an asterisk. Note that relative peak areas cannot be used to compare relative quantities of two different metabolites. *P* and *q* values < 0.05 are shown in ***bold***.

	Healthy		Chronic Enteropathy			
Compound	**Median**	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
1,2-propanediol	0.4667	[0.2553-2.8892]	1.1693	[0.2553-8.8776]	**0.0263**	0.0929
1,3-diaminopropane	0.2594	[0.2594-1.5561]	0.2594	[0.2594-39.101]	0.8545	0.9174
1,5-anhydroglucitol (1,5-AG)	0.1785	[0.1221-1.1503]	1.7475	[0.3162-11.03]	**0.0000**	**0.0016**
10-heptadecenoate (17:1n7)	0.8657	[0.3247-3.6391]	1.8169	[0.1717-17.8203]	0.2998	0.4805
10-hydroxystearate	0.7473	[0.2144-2.6944]	2.8566	[0.2648-17.2784]	**0.0062**	**0.0399**
10-nonadecenoate (19:1n9)	0.9752	[0.3434-2.9329]	1.5884	[0.2485-15.203]	0.5338	0.6876
12,13-DiHOME	1.4844	[0.4996-3.03]	0.8188	[0.0698-5.1941]	0.1057	0.2391
12-dehydrocholate	0.6052	[0.1986-33.2721]	0.5097	[0.1591-41.145]	0.1957	0.3641
12-hydroxyoleate	0.4341	[0.2407-4.6698]	4.0735	[0.1492-37.2416]	**0.0037**	**0.0269**
13-HODE + 9-HODE	0.8219	[0.3762-1.7473]	1.1002	[0.0084-5.7011]	0.5614	0.7115
13-methylmyristic acid	1.6836	[0.1132-9.3985]	0.3864	[0.0845-4.7114]	**0.0032**	**0.0254**
15-methylpalmitate (isobar with 2-methylpalmitate)	0.9914	[0.5009-2.3236]	1.0314	[0.4135-3.0176]	0.9339	0.9582
17-methylstearate	0.9301	[0.5482-1.931]	1.3120	[0.2498-6.2635]	0.3615	0.5391
19,20-DiHDPA	0.8868	[0.0363-4.756]	1.0202	[0.0401-4.0574]	0.7089	0.8229
1-arachidonylglycerol	0.6680	[0.2263-2.4342]	1.7541	[0.2263-8.7227]	0.0796	0.1955
1-docosahexaenoylglycerol	0.6631	[0.1582-3.1366]	0.1582	[0.1582-41.1925]	0.3242	0.5093
1H-quinolin-2-one	0.8822	[0.1634-28.4684]	0.1634	[0.1634-0.1634]	**0.0000**	**0.0014**
1-linolenoylglycerol	1.1211	[0.2121-53.1655]	0.3978	[0.0806-14.7761]	0.1844	0.3447
1-linoleoylglycerol (1-monolinolein)	2.3704	[0.359-7.7933]	0.4910	[0.0843-38.8233]	**0.0279**	0.0947
1-linoleoylglycerophosphocholine (18:2n6)	0.9217	[0.1581-6.9648]	0.5225	[0.1581-13.8959]	0.4637	0.6303
1-linoleoylglycerophosphoethanolamine*	0.0909	[0.0909-1.2573]	0.0909	[0.0909-15.0424]	0.6211	0.7555
1-margaroylglycerophosphocholine (17:0)	0.2529	[0.2529-0.3741]	0.2529	[0.2529-8.6859]	0.1418	0.2928
1-margaroylglycerophosphoethanolamine*	0.4830	[0.3727-1.9014]	0.3727	[0.3727-1.4264]	0.1167	0.2601
1-methyladenine	0.6844	[0.1849-1.9713]	1.4744	[0.3369-13.9368]	**0.0079**	**0.0453**
1-methyl-beta-carboline-3-carboxylic acid	1.0000	[0.2997-2.7202]	0.7815	[0.0667-57.4431]	0.6482	0.7729
1-methylimidazoleacetate	1.0752	[0.0613-12.6798]	0.2249	[0.0471-6.8776]	0.0614	0.1665
1-methylnicotinamide	1.0580	[0.1062-3.5365]	0.6903	[0.1062-3.7644]	0.5067	0.6616
1-methylurate	1.1291	[0.3126-6.3497]	0.5203	[0.1035-2.9213]	**0.0181**	0.0758
1-methylxanthine	1.0171	[0.5129-1.8813]	0.9829	[0.0613-3.5209]	0.5614	0.7115
1-myristoylglycerol (1-monomyristin)	1.9608	[0.4641-4.5369]	0.6474	[0.3122-21.6146]	0.1057	0.2391
1-oleoylglycerol (1-monoolein)	1.3800	[0.2446-6.0364]	0.7736	[0.0648-12.7071]	**0.0465**	0.1370
1-oleoylglycerophosphocholine (18:1)	1.0486	[0.0728-2.2969]	0.5123	[0.0728-9.8204]	0.9337	0.9582
1-oleoylglycerophosphoethanolamine	0.3032	[0.3032-2.5759]	0.4447	[0.3032-61.6865]	0.3197	0.5032
1-oleoylglycerophosphoglycerol*	0.2073	[0.2073-1.0651]	0.2073	[0.2073-33.5241]	0.1599	0.3130
1-palmitoylglycerol (1-monopalmitin)	1.0991	[0.3657-2.8696]	0.8546	[0.2894-10.3596]	0.5614	0.7115
1-palmitoylglycerophosphocholine (16:0)	0.9212	[0.0376-4.3477]	1.0677	[0.1428-16.1569]	0.4553	0.6205
1-palmitoylglycerophosphoethanolamine	1.4794	[0.0345-6.611]	0.7012	[0.0345-53.5511]	0.8519	0.9159
1-palmitoylglycerophosphoglycerol*	1.0000	[0.0838-3.4259]	0.7338	[0.0838-9.6563]	0.7241	0.8381
1-palmitoylglycerophosphoinositol*	0.2801	[0.2801-8.914]	0.2801	[0.2801-23.6638]	0.7871	0.8788
1-palmitoylplasmenylethanolamine*	0.5841	[0.0864-3.2301]	0.0864	[0.0864-34.1766]	0.3294	0.5154
1-pentadecanoylglycerol (1-monopentadecanoin)	1.5973	[0.3488-7.2626]	0.3690	[0.1019-10.3443]	**0.0070**	**0.0418**
1-stearoylglycerol (1-monostearin)	0.9049	[0.3718-7.0455]	1.1804	[0.5088-6.483]	0.0512	0.1472
1-stearoylglycerophosphocholine (18:0)	0.3100	[0.0788-1.7794]	1.1974	[0.0788-18.8548]	**0.0249**	0.0894
1-stearoylglycerophosphoethanolamine	0.5008	[0.0839-5.5339]	2.6153	[0.1518-76.6895]	**0.0250**	0.0894
1-stearoylglycerophosphoglycerol	0.2552	[0.2552-1.2036]	0.2552	[0.2552-56.701]	0.5902	0.7349
1-stearoylglycerophosphoinositol	0.0868	[0.0868-1.1211]	0.7037	[0.0868-28.8554]	**0.0004**	**0.0074**
1-stearoylglycerophosphoserine*	0.0769	[0.0769-1.5421]	0.8583	[0.0769-89.0432]	**0.0021**	**0.0203**
1-stearoylplasmenylethanolamine*	0.5504	[0.0263-3.8286]	1.2426	[0.0136-28.3806]	0.3195	0.5032
2-(4-hydroxyphenyl)propionate	0.3887	[0.3887-63.8739]	0.3887	[0.3887-2.0746]	0.4714	0.6386

Compound	Healthy Median	Healthy Range	Chronic Enteropathy Median	Chronic Enteropathy Range	*p*-value	*q*-value
2,3-dihydroxyisovalerate	1.0811	[0.2039-7.5345]	0.8839	[0.2369-29.4536]	0.8357	0.9047
2,4,6-trihydroxybenzoate	1.0000	[0.0929-4.853]	0.8609	[0.0929-5.2584]	0.5755	0.7270
2,8-quinolinediol	1.0046	[0.2049-1.7682]	0.3672	[0.0817-28.2229]	0.1834	0.3447
2-[(2-methylbutanoyl)amino]-3-phenylpropanoic acid	0.6127	[0.0639-2.8015]	0.0639	[0.0639-13.0458]	**0.0271**	0.0947
2-aminoadipate	0.9194	[0.5356-1.9858]	1.0411	[0.0545-8.5154]	0.4068	0.5810
2-aminobutyrate	0.7071	[0.2038-3.4973]	1.0875	[0.0382-6.6831]	0.3615	0.5391
2-aminoheptanoate	1.1035	[0.4323-2.1032]	0.7226	[0.3433-3.0276]	0.0677	0.1790
2-aminophenol sulfate	0.2424	[0.2424-54.0926]	0.2424	[0.2424-0.3287]	0.2722	0.4656
2-arachidonoylglycerophosphocholine*	0.1231	[0.1231-1.5822]	0.1231	[0.1231-3.1826]	0.1789	0.3400
2'-deoxyadenosine	1.1742	[0.2473-3.9313]	0.8639	[0.1503-3.9407]	0.1711	0.3276
2'-deoxycytidine	1.1410	[0.122-4.4011]	0.3400	[0.122-4.507]	0.0951	0.2254
2'-deoxyguanosine	1.1882	[0.1992-6.4138]	0.5369	[0.1992-13.3477]	0.4296	0.5971
2'-deoxyinosine	2.5708	[0.3025-5.4587]	0.3310	[0.0248-6.5178]	**0.0251**	0.0894
2-deoxyribose	0.6832	[0.2466-2.7388]	0.9877	[0.2466-2.7266]	0.9668	0.9743
2'-deoxyuridine	1.8333	[0.4815-3.6029]	0.7271	[0.0123-2.5173]	0.1844	0.3447
2-hydroxy-3-methylvalerate	0.1679	[0.0267-2.3425]	1.8852	[0.5855-77.0248]	**0.0001**	**0.0033**
2-hydroxyadipate	1.1766	[0.5869-4.0341]	0.5604	[0.1053-3.2367]	**0.0225**	0.0848
2-hydroxybutyrate (AHB)	0.2178	[0.1112-10.714]	5.1633	[0.3311-15.1281]	**0.0014**	**0.0165**
2-hydroxydecanoate	0.9990	[0.6786-1.1992]	1.0037	[0.435-12.5549]	0.9010	0.9379
2-hydroxyglutarate	0.5101	[0.1666-2.0226]	1.9907	[0.276-8.3645]	**0.0005**	**0.0076**
2-hydroxyhippurate (salicylurate)	0.6347	[0.177-1.3921]	1.2533	[0.177-10.0059]	**0.0135**	0.0651
2-hydroxyoctanoate	1.2024	[0.5839-2.4701]	0.7938	[0.1323-1.8586]	0.1464	0.2981
2-hydroxypalmitate	0.7100	[0.1929-2.9837]	2.0140	[0.0942-11.7271]	**0.0225**	0.0848
2-hydroxystearate	0.8077	[0.2525-3.7748]	2.0375	[0.0022-12.5642]	0.0971	0.2254
2-isopropylmalate	1.5852	[0.1903-7.2482]	0.8599	[0.0204-3.7352]	0.1710	0.3276
2-linoleoylglycerol (2-monolinolein)	1.3878	[0.2864-6.0051]	0.6906	[0.1028-27.2382]	0.1057	0.2391
2-linoleoylglycerophosphocholine*	0.1134	[0.1134-1.9952]	0.1134	[0.1134-4.0572]	0.4259	0.5971
2-linoleoylglycerophosphoethanolamine*	0.3997	[0.3997-2.5343]	0.3997	[0.3997-1.9468]	0.3616	0.5391
2-methylbutyrylcarnitine (C5)	0.5167	[0.362-1.9693]	1.0000	[0.362-15.4418]	0.0554	0.1563
2-myristoylglycerol (2-monomyristin)	1.1125	[0.0019-4.6991]	0.5693	[0.0019-45.5701]	0.9669	0.9743
2-oleoylglycerol (2-monoolein)	0.9976	[0.2832-5.2886]	1.0024	[0.0761-12.6461]	0.4068	0.5810
2-oleoylglycerophosphocholine*	0.4633	[0.4633-2.0785]	0.4633	[0.4633-3.9899]	0.2300	0.4087
2-oleoylglycerophosphoethanolamine*	0.2193	[0.2193-1.9934]	0.2193	[0.2193-4.4772]	0.2878	0.4779
2-oxindole-3-acetate	7.2500	[1.7082-24.8117]	0.1157	[0.001-0.2918]	**0.0000**	**0.0011**
2-palmitoylglycerol (2-monopalmitin)	0.8738	[0.0833-3.3348]	1.5686	[0.3164-9.2684]	0.3615	0.5391
2-palmitoylglycerophosphocholine*	0.2394	[0.2394-1.1482]	0.7022	[0.2394-5.5209]	0.0791	0.1955
2-palmitoylglycerophosphoethanolamine*	0.1158	[0.1158-1.5752]	0.1158	[0.1158-19.5806]	0.5060	0.6616
2-pentanamido-3-phenylpropanoic acid	0.2951	[0.0584-3.3269]	0.0584	[0.0584-2.9738]	**0.0437**	0.1322
2-piperidinone	1.1756	[0.1113-2.0532]	0.0389	[0.0093-2.5789]	**0.0251**	0.0894
2-stearoylglycerol (2-monostearin)	0.9659	[0.1645-2.997]	1.0534	[0.2565-8.4386]	0.4306	0.5971
2-stearoylglycerophosphocholine*	0.1529	[0.1529-1.3698]	0.5348	[0.1529-19.2919]	**0.0133**	0.0646
2-stearoylglycerophosphoethanolamine*	0.3489	[0.3489-1]	0.3489	[0.3489-42.1489]	0.2989	0.4805
3-(3-hydroxyphenyl)propionate	1.5807	[0.1228-7612.0503]	0.5119	[0.1228-12.3101]	**0.0084**	**0.0470**
3-(4-hydroxyphenyl)lactate	0.7342	[0.0317-13.2026]	1.2227	[0.2201-27.1103]	0.0971	0.2254
3-(4-hydroxyphenyl)propionate	1.5688	[0.9916-3.8932]	0.1288	[0.0228-1.3382]	**0.0000**	**0.0014**
3,4-dihydroxybenzoate	1.5210	[0.0436-3.5905]	0.0436	[0.0436-1.9118]	**0.0006**	**0.0087**
3,4-dihydroxyphenylacetate	0.4836	[0.0723-61.4618]	0.0723	[0.0723-5.1981]	0.2874	0.4779
3,7-dimethylurate	0.0441	[0.0441-0.0441]	0.0441	[0.0441-18.8239]	0.0797	0.1955
3-[3-(sulfooxy)phenyl]propanoic acid	0.3052	[0.3052-7.6935]	0.3052	[0.3052-0.3052]	**0.0384**	0.1195
3-aminoisobutyrate	0.5899	[0.1493-1.6751]	0.1493	[0.1493-2.9389]	0.1392	0.2883
3beta,7alpha-dihydroxy-5-cholestenoate	0.0418	[0.0418-1.857]	0.0418	[0.0418-11.5644]	0.6547	0.7784
3beta,7beta-dihydroxy-5-cholestenoate	1.2262	[0.4758-3.2571]	0.6884	[0.1321-4.0307]	0.0815	0.1986
3b-hydroxy-5-cholenoic acid	1.0979	[0.3965-4.0082]	0.5253	[0.0099-3.8849]	0.0744	0.1904
3-dehydrocholate	1.0053	[0.3271-3.101]	0.9872	[0.1451-16.178]	0.6783	0.7979

Compound	Healthy		Chronic Enteropathy			
	Median	Range	Median	Range	*p*-value	*q*-value
3-hydroxy-2-ethylpropionate	1.3124	[0.3671-2.7351]	0.3265	[0.2214-2.5026]	**0.0018**	**0.0197**
3-hydroxy-3-methylglutarate	1.0631	[0.2698-7.3912]	0.8577	[0.1474-4.947]	0.3837	0.5613
3-hydroxybutyrate (BHBA)	1.5644	[0.2652-3.7018]	0.4424	[0.0933-10.4478]	**0.0225**	0.0848
3-hydroxydecanoate	0.8091	[0.5669-1.7815]	1.1763	[0.6963-2.7713]	**0.0144**	0.0670
3-hydroxyindolin-2-one	1.2202	[0.2767-2.2848]	0.4382	[0.0113-2.2775]	**0.0279**	0.0947
3-hydroxyisobutyrate	0.8289	[0.1413-1.8225]	0.6354	[0.1413-6.1242]	0.4920	0.6552
3-hydroxylaurate	0.8419	[0.2887-2.7577]	1.0058	[0.4425-4.9833]	0.3401	0.5248
3-hydroxymyristate	1.0400	[0.5356-3.7634]	0.9634	[0.2134-3.608]	0.7089	0.8229
3-hydroxyoctanoate	1.2300	[0.6242-2.0276]	0.7853	[0.4574-3.4191]	**0.0344**	0.1092
3-hydroxypalmitate	2.1569	[0.12-3.4192]	0.3164	[0.1148-2.2036]	**0.0016**	**0.0180**
3-hydroxypropanoate	0.8109	[0.3008-7.9955]	1.3533	[0.5799-33.5356]	**0.0421**	0.1285
3-hydroxysebacate	1.1263	[0.4369-2.8894]	0.5529	[0.1464-5.4539]	**0.0070**	**0.0418**
3-indoxyl sulfate	0.1367	[0.1367-3.4531]	0.1367	[0.1367-13.9539]	0.5325	0.6876
3-methoxytyrosine	1.2356	[0.3982-5.6838]	0.6420	[0.0857-5.4367]	0.0511	0.1472
3-methyl-2-oxobutyrate	0.7690	[0.2516-1.7975]	1.6847	[0.3051-56.5531]	**0.0181**	0.0758
3-methyl-2-oxovalerate	1.2440	[0.0774-4.8045]	0.8750	[0.0042-4.1933]	0.5069	0.6616
3-methylglutarylcarnitine (1)	0.1537	[0.1537-1.3381]	0.3098	[0.1537-23.8941]	0.0957	0.2254
3-methylhistidine	1.0538	[0.0856-8.8067]	0.3392	[0.0856-4.4918]	0.1184	0.2632
3-phenylpropionate (hydrocinnamate)	1.6446	[0.0348-2.5737]	0.2405	[0.0112-5.8583]	**0.0224**	0.0848
3-ureidopropionate	2.0348	[0.0916-19.6466]	0.3691	[0.0144-6.6725]	**0.0197**	0.0811
4-acetamidobutanoate	1.0000	[0.1997-2.4289]	0.1997	[0.1997-6.6954]	**0.0384**	0.1195
4-acetylphenol sulfate	0.0797	[0.0797-1.4681]	0.0797	[0.0797-1.5755]	0.6093	0.7537
4-guanidinobutanoate	2.1359	[0.5179-3.9682]	0.5253	[0.1113-4.2172]	**0.0161**	0.0702
4-hydroxybenzoate	2.6652	[0.8558-6.9585]	0.2255	[0.0556-1.1442]	**0.0000**	**0.0011**
4-hydroxybenzyl alcohol	0.5124	[0.5124-1.5488]	0.5124	[0.5124-2.9149]	0.4537	0.6205
4-hydroxycinnamate	1.3574	[0.36-5.4185]	0.5290	[0.0752-7.6666]	**0.0309**	0.1009
4-hydroxycyclohexylcarboxylic acid	0.4067	[0.4067-10.1202]	0.4067	[0.4067-1.9403]	0.9527	0.9724
4-hydroxyphenylacetate	1.2103	[0.3351-4.1877]	0.1135	[0.0132-6.3263]	**0.0310**	0.1009
4-hydroxyphenylacetyl glycine	0.2689	[0.0139-1.3309]	1.3631	[0.0139-88.07]	**0.0025**	**0.0217**
4-hydroxyphenylpyruvate	1.3259	[0.1983-5.0466]	0.9298	[0.0334-6.7083]	0.8357	0.9047
4-imidazoleacetate	1.0908	[0.488-1.7651]	0.5584	[0.0529-1.8206]	**0.0344**	0.1092
4-methyl-2-oxopentanoate	1.0001	[0.1253-3.3018]	0.9999	[0.0162-5.4733]	0.8035	0.8856
4-methylcatechol sulfate	0.2972	[0.2972-1]	0.2972	[0.2972-6.9609]	0.2925	0.4805
4-methylthio-2-oxobutanoate	0.9324	[0.1239-3.7721]	0.7943	[0.1239-22.6672]	0.9004	0.9379
4-ureidobutyrate	1.4711	[0.0809-4.6092]	0.0809	[0.0809-1]	**0.0000**	**0.0026**
5-(2-Hydroxyethyl)-4-methylthiazole	1.6959	[0.4157-5.6302]	0.1111	[0.0242-8.6919]	**0.0025**	**0.0217**
5-(galactosylhydroxy)-L-lysine	0.1089	[0.1089-0.1089]	0.1089	[0.1089-50.6784]	**0.0036**	**0.0269**
5,6-DiHETrE	0.8475	[0.1113-2.4162]	1.0252	[0.1113-8.933]	0.4304	0.5971
5,6-dihydrothymine	0.5825	[0.1096-2.165]	1.2970	[0.1096-5.2305]	0.0926	0.2222
5,6-dihydrouracil	0.9335	[0.004-3.4577]	0.8348	[0.004-3.3564]	0.9338	0.9582
5-aminovalerate	0.9328	[0.2694-2.2728]	1.0672	[0.0104-5.9787]	0.8035	0.8856
5-dodecenoate (12:1n7)	1.0294	[0.3943-1.4453]	0.7226	[0.1102-2.8643]	0.9010	0.9379
5-HETE	0.6935	[0.1095-2.5444]	0.8215	[0.1095-23.7421]	0.6165	0.7537
5-hydroxydecanoate	0.8988	[0.1629-3.3894]	0.1629	[0.1629-143.2411]	**0.0295**	0.0993
5-hydroxyindoleacetate	1.8408	[0.6563-4.3699]	0.4321	[0.1281-2.8119]	**0.0005**	**0.0076**
5-hydroxylysine	0.7608	[0.0789-6.6019]	1.6444	[0.0376-7.1045]	0.3614	0.5391
5-hydroxymethyluracil	0.8608	[0.0972-2.4976]	0.2652	[0.0972-3.0122]	0.3371	0.5248
5-ketogluconate	0.5655	[0.2996-5.5536]	1.7324	[0.1767-61.2063]	**0.0465**	0.1370
5-methyl-2'-deoxycytidine	1.1478	[0.5516-2.2823]	0.5796	[0.066-5.1624]	**0.0462**	0.1370
5-methylthioadenosine (MTA)	0.3586	[0.3586-1.4354]	0.3586	[0.3586-5.9853]	0.0752	0.1908
5-methyluridine (ribothymidine)	1.6743	[0.2146-3.2062]	0.0573	[0.029-9.9821]	**0.0242**	0.0894
5-oxoproline	0.5854	[0.1273-1.7887]	2.4828	[0.5204-13.8445]	**0.0001**	**0.0043**
6-hydroxydaidzein	0.9170	[0.0816-59.8135]	0.0816	[0.0816-16.9609]	0.0761	0.1913
6-hydroxynicotinate	1.0409	[0.0909-1.9128]	0.0745	[0.0745-1.5235]	**0.0003**	**0.0059**

Compound	Healthy Median	Healthy Range	Chronic Enteropathy Median	Chronic Enteropathy Range	*p*-value	*q*-value
6-oxolithocholate	1.2730	[0.175-4.5981]	0.5368	[0.0128-29.0662]	0.1056	0.2391
6-oxopiperidine-2-carboxylic acid	0.9707	[0.4274-1.6352]	1.1434	[0.4545-8.2232]	0.2998	0.4805
7,12-diketolithocholate	2.0607	[0.0823-12.1295]	0.1203	[0.0823-14.6585]	**0.0023**	**0.0217**
7-alpha-hydroxy-3-oxo-4-cholestenoate	0.7719	[0.0271-2.4202]	1.1176	[0.0271-46.277]	0.4296	0.5971
7-ketodeoxycholate	0.6697	[0.2139-14.6467]	1.2832	[0.1494-23.5424]	0.2998	0.4805
7-ketolithocholate	0.3737	[0.0793-3.1325]	1.1615	[0.0793-7.4681]	0.0739	0.1904
7-methylguanine	1.1855	[0.642-2.7987]	0.7538	[0.1301-2.056]	**0.0161**	0.0702
7-methylurate	0.5017	[0.0038-2.3183]	0.1540	[0.0038-11.8759]	0.2952	0.4805
7-nonadecenoate (19:1n12)	0.7337	[0.3167-4.6204]	4.6224	[0.1465-39.7403]	**0.0144**	0.0670
9,10-DiHOME	1.2797	[0.2969-2.9372]	0.7729	[0.3686-3.8945]	0.7400	0.8416
acetoacetate	1.9505	[0.2873-4.6225]	0.5185	[0.2797-2.0231]	**0.0016**	**0.0180**
acetylcarnitine	0.7844	[0.0533-4.9477]	1.4625	[0.018-16.7198]	0.4807	0.6423
aconitate [cis or trans]	0.9766	[0.5228-1.6098]	1.4812	[0.3539-7.0654]	0.1248	0.2670
adenine	1.0688	[0.4584-4.0017]	0.6487	[0.0928-3.0876]	0.2998	0.4805
adenosine	1.8275	[0.5424-5.7513]	0.6632	[0.1033-6.5215]	**0.0279**	0.0947
adenosine 5'-monophosphate (AMP)	0.0644	[0.0644-0.0644]	0.0644	[0.0644-16.2868]	0.0797	0.1955
adrenate (22:4n6)	0.8896	[0.2378-1.655]	1.7575	[0.1088-13.9074]	0.1249	0.2670
agmatine	0.4638	[0.0598-5.265]	1.5190	[0.0598-21.8349]	**0.0278**	0.0947
alanine	0.7452	[0.3411-1.9062]	1.6500	[0.5119-7.642]	**0.0128**	0.0627
alanylalanine	0.7819	[0.2022-2.9228]	1.0809	[0.1559-2.6833]	0.8682	0.9196
alanylarginine	1.1765	[0.161-5.8734]	0.5514	[0.0937-8.6664]	0.1584	0.3118
alanylisoleucine	1.1320	[0.1012-6.8725]	0.9949	[0.0363-5.5799]	0.8357	0.9047
alanylleucine	1.2489	[0.1188-4.1307]	0.7110	[0.3163-4.1449]	0.6187	0.7537
alanylmethionine	1.2418	[0.0819-4.5169]	0.6593	[0.0819-6.2978]	0.3725	0.5541
alanylphenylalanine	1.0629	[0.1041-5.0153]	0.9371	[0.1472-5.8301]	0.7400	0.8416
alanylproline	0.9006	[0.2998-3.4327]	1.0334	[0.4062-4.737]	0.2455	0.4265
alanyltryptophan	1.2200	[0.0552-2.2381]	0.9809	[0.2614-3.9274]	0.3837	0.5613
alanyltyrosine	0.9080	[0.1953-4.1517]	1.2009	[0.2141-5.7649]	0.9010	0.9379
alanylvaline	0.9876	[0.2406-6.6118]	1.0105	[0.1114-6.3947]	0.7089	0.8229
allantoic acid	0.0700	[0.07-2.2107]	0.1371	[0.07-17.1249]	**0.0245**	0.0894
allantoin	0.0222	[0.0093-13.6238]	3.8543	[0.0093-23.6102]	**0.0076**	**0.0447**
allo-isoleucine	0.3533	[0.1195-7.2377]	1.0221	[0.1195-8.5179]	0.2876	0.4779
alpha-CEHC	1.9940	[0.0648-7.2444]	0.1096	[0.0186-3.9441]	**0.0090**	**0.0487**
alpha-CEHC glucuronide*	0.0148	[0.0148-0.0148]	0.0148	[0.0148-16.0546]	**0.0036**	**0.0269**
alpha-CEHC sulfate	0.3669	[0.0039-15.0232]	1.0797	[0.047-8.0483]	0.3195	0.5032
alpha-glutamylglutamate	0.5179	[0.0856-3.2413]	1.6040	[0.4082-8.0655]	**0.0054**	**0.0363**
alpha-glutamylthreonine	0.4609	[0.0841-2.9068]	1.4696	[0.0841-4.7593]	0.1842	0.3447
alpha-glutamyltryptophan	0.8679	[0.0806-3.9572]	0.9608	[0.0806-2.8707]	0.9009	0.9379
alpha-glutamyltyrosine	1.6394	[0.2667-4.4116]	0.9970	[0.0585-4.409]	0.4068	0.5810
alpha-glutamylvaline	0.2909	[0.023-4.7125]	1.0616	[0.023-3.2814]	0.4304	0.5971
alpha-hydroxyisocaproate	0.1060	[0.0118-2.5201]	2.1760	[0.2716-43.0681]	**0.0002**	**0.0050**
alpha-hydroxyisovalerate	0.2204	[0.0027-3.445]	1.7462	[0.4065-29.6122]	**0.0021**	**0.0203**
alpha-ketoglutarate	0.5729	[0.2152-1.6455]	3.0621	[0.4068-169.4707]	**0.0002**	**0.0055**
alpha-muricholate	1.7248	[0.2885-5.5636]	0.3344	[0.007-8.721]	**0.0225**	0.0848
alpha-tocopherol	1.5622	[0.2672-3.5291]	0.7414	[0.0309-5.2896]	**0.0465**	0.1370
alpha-tocopherol acetate	1.2600	[0.3504-5.6863]	0.7870	[0.031-3.3294]	0.1585	0.3118
anserine	1.2689	[0.2819-3.3222]	0.1062	[0.0038-5.326]	**0.0344**	0.1092
anthranilate	0.9594	[0.14-2.1298]	1.0066	[0.5483-2.5014]	0.2808	0.4703
apigenin	1.7438	[0.1027-11.2783]	0.4851	[0.0157-4.0591]	**0.0225**	0.0848
arabinose	1.8066	[0.3971-8.5153]	0.5435	[0.0111-7.1108]	**0.0090**	**0.0487**
arabitol	0.1112	[0.1112-0.7734]	1.0860	[0.1112-2.2283]	**0.0101**	0.0539
arabonate	0.1011	[0.1011-0.5844]	0.9747	[0.1011-6.0083]	**0.0025**	**0.0217**
arachidonate (20:4n6)	0.5657	[0.1873-2.4479]	1.7338	[0.0919-32.8549]	**0.0079**	**0.0453**
arachidonoyl ethanolamide	0.6608	[0.1498-2.5003]	1.9819	[0.1498-28.7656]	0.1247	0.2670

Compound	Healthy		Chronic Enteropathy			
	Median	Range	Median	Range	*p*-value	*q*-value
arginine	0.7144	[0.2792-3.8006]	1.5969	[0.3033-37.4299]	0.0971	0.2254
arginylaspartate	0.2278	[0.0618-3.053]	0.9598	[0.0618-4.7195]	0.4168	0.5932
arginylisoleucine	1.0973	[0.0606-6.3947]	0.7024	[0.0606-7.3343]	0.4798	0.6423
arginylleucine	1.7484	[0.1105-7.4564]	0.6700	[0.1165-3.3575]	0.1711	0.3276
arginylphenylalanine	1.3247	[0.102-3.2462]	0.3780	[0.0269-2.8748]	**0.0251**	0.0894
arginylproline	0.8328	[0.0652-3.4103]	1.0838	[0.0652-3.7428]	0.4679	0.6349
arginyltyrosine	0.8899	[0.1117-3.7668]	0.1117	[0.1117-3.6241]	**0.0376**	0.1184
arginylvaline	1.1562	[0.0263-4.2257]	0.3493	[0.0263-2.8302]	0.1240	0.2670
asparagine	0.3107	[0.0786-6.5284]	1.1592	[0.0995-23.5462]	0.1585	0.3118
asparagylisoleucine	1.4277	[0.2456-7.2706]	0.8008	[0.0012-3.0265]	0.0620	0.1665
asparagylleucine	1.4247	[0.1755-5.9738]	0.7208	[0.0416-5.075]	0.1249	0.2670
asparagylvaline	1.2801	[0.219-5.493]	0.9692	[0.0142-4.2607]	0.9010	0.9379
aspartate	0.5466	[0.1578-5.567]	3.3042	[0.0208-27.6348]	**0.0070**	**0.0418**
aspartate-glutamate	0.1999	[0.0353-2.0599]	4.5822	[0.0353-114.1268]	**0.0005**	**0.0076**
aspartylaspartate	0.0350	[0.035-2.0785]	1.3617	[0.0671-48.7688]	**0.0003**	**0.0066**
aspartylglutamine	0.5411	[0.0396-2.0445]	2.5976	[0.2261-54.215]	**0.0007**	**0.0098**
aspartylglycine	0.3104	[0.0837-1.743]	2.4142	[0.4214-51.1879]	**0.0002**	**0.0050**
aspartylleucine	0.8578	[0.0664-3.1413]	1.3014	[0.203-3.3992]	**0.0465**	0.1370
aspartylphenylalanine	0.5666	[0.0699-2.4851]	1.2184	[0.104-3.6034]	**0.0161**	0.0702
aspartyltryptophan	0.3992	[0.1222-5.2437]	0.9635	[0.1222-26.2187]	0.1036	0.2376
aspartylvaline	0.7006	[0.0275-8.0736]	1.1359	[0.0275-4.3693]	0.2806	0.4703
assymetric dimethylarginine (ADMA)	0.1091	[0.0187-2.7263]	1.1957	[0.0187-3.9081]	**0.0155**	0.0702
azelate (nonanedioate)	2.2805	[0.8211-4.6528]	0.6810	[0.2465-1.8503]	**0.0004**	**0.0074**
benzoate	1.1494	[0.596-1.8453]	0.8195	[0.2756-1.8197]	**0.0344**	0.1092
beta-alanine	2.5415	[0.5219-11.2159]	0.4525	[0.1067-2.2257]	**0.0018**	**0.0197**
beta-hydroxyisovalerate	1.5632	[0.42-3.3714]	0.1332	[0.0276-1.2738]	**0.0001**	**0.0032**
beta-hydroxyisovaleroylcarnitine	0.0390	[0.039-3.4351]	0.9237	[0.039-8.5661]	**0.0008**	**0.0104**
betaine	0.5621	[0.2984-11.8053]	1.0844	[0.2984-34.9215]	0.0744	0.1904
beta-muricholate	0.4508	[0.0449-6.2919]	2.2600	[0.0256-40.1661]	**0.0381**	0.1194
beta-sitosterol	1.8184	[0.7775-2.4802]	0.6002	[0.1567-1.9787]	**0.0001**	**0.0028**
beta-tocopherol	0.4461	[0.4461-1]	0.4461	[0.4461-4.9468]	0.3274	0.5133
bilirubin (E,E)*	0.0424	[0.0424-1.1561]	0.0424	[0.0424-5.5441]	0.2061	0.3789
bilirubin (Z,Z)	0.1129	[0.1129-1.1607]	0.8561	[0.1129-9.1417]	**0.0065**	**0.0415**
biliverdin	1.0000	[0.094-3.2933]	0.7108	[0.094-3.0431]	0.8681	0.9196
biocytin	0.8395	[0.0728-3.9601]	1.0000	[0.0728-5.4279]	0.7713	0.8650
biopterin	0.7098	[0.0217-1.5828]	1.3140	[0.0217-3.8197]	0.0883	0.2138
biotin	0.5099	[0.0447-26.3799]	2.2367	[0.0274-20.6366]	**0.0114**	0.0579
butyrylcarnitine	0.0389	[0.0389-2.0284]	0.0389	[0.0389-3.5163]	0.7138	0.8273
butyrylglycine	0.3418	[0.0791-2.251]	1.0561	[0.0791-6.905]	0.0643	0.1722
cadaverine	0.4212	[0.0058-50.1027]	5.8839	[0.0606-80.8022]	**0.0144**	0.0670
campesterol	1.1399	[0.6302-1.6244]	0.5878	[0.1136-3.6083]	**0.0144**	0.0670
caprate (10:0)	0.9924	[0.5286-4.844]	1.0076	[0.4141-47.1251]	1.0000	1.0000
caproate (6:0)	1.1306	[0.8842-1.579]	0.8333	[0.5517-1.6058]	**0.0114**	0.0579
caprylate (8:0)	1.1683	[0.508-3.3773]	0.8797	[0.536-7.4669]	0.2134	0.3851
carboxyethyl-GABA*	1.4271	[0.8138-3.0233]	0.7591	[0.1582-1.3938]	**0.0005**	**0.0076**
carnitine	1.3973	[0.0797-4.747]	0.8425	[0.1365-3.0471]	0.3837	0.5613
carnosine	0.8435	[0.1053-4.554]	0.9652	[0.1053-17.0716]	0.9667	0.9743
catechin	0.3469	[0.3469-10.1136]	0.3469	[0.3469-1.3967]	0.1599	0.3130
catechol sulfate	0.2463	[0.2463-4.1406]	0.2463	[0.2463-5.5278]	0.7079	0.8229
C-glycosyltryptophan*	0.0486	[0.0486-0.3841]	0.7875	[0.0486-8.0044]	**0.0039**	**0.0277**
chenodeoxycholate	0.1269	[0.1269-7.901]	0.9494	[0.1269-7.1643]	**0.0101**	0.0539
chiro-inositol	0.0896	[0.0896-72.3084]	0.7064	[0.0896-49.155]	0.2874	0.4779
chlorogenate	0.0888	[0.0888-0.0888]	0.0888	[0.0888-26.1041]	0.0797	0.1955
cholate	0.3267	[0.0334-4.8596]	2.8427	[0.0816-10.4469]	**0.0011**	**0.0131**

Compound	Healthy Median	Healthy Range	Chronic Enteropathy Median	Chronic Enteropathy Range	*p*-value	*q*-value
cholestanol	0.9656	[0.4888-1.4335]	1.0239	[0.3637-5.6793]	0.6185	0.7537
cholesterol	0.9279	[0.4084-1.2679]	1.7093	[0.3399-4.8153]	**0.0421**	0.1285
choline	0.8655	[0.2248-1.2195]	1.6657	[0.6872-7.9863]	**0.0028**	**0.0234**
choline phosphate	0.5659	[0.1449-1.4237]	0.1449	[0.1449-322.7082]	0.7428	0.8436
chrysoeriol	1.7540	[0.0158-10.4908]	0.6311	[0.0158-2.617]	0.2193	0.3940
cinnamoylglycine	0.4628	[0.4628-0.471]	0.4628	[0.4628-16.5925]	0.2615	0.4503
cis-Cyclo[L-ala-L-Pro]	0.8571	[0.4221-1.6806]	1.3242	[0.4221-4.0689]	**0.0250**	0.0894
cis-urocanate	0.9969	[0.3184-4.6541]	1.0161	[0.2907-4.8037]	0.7400	0.8416
cis-vaccenate (18:1n7)	0.9692	[0.2998-2.2746]	1.3536	[0.0455-6.2398]	0.1585	0.3118
citramalate	0.8336	[0.2384-2.9241]	0.7080	[0.2384-15.1119]	0.8674	0.9196
citrate	0.6492	[0.2852-2.3137]	2.3996	[0.2852-10.4133]	0.0848	0.2061
citrulline	0.8165	[0.0413-3.2737]	1.1835	[0.0911-4.2169]	0.6482	0.7729
coprostanol	0.6330	[0.2057-3.7535]	0.2057	[0.2057-1.8682]	0.0738	0.1904
creatine	1.0259	[0.0024-2.0902]	0.9898	[0.1277-4.9274]	0.2998	0.4805
creatinine	1.0125	[0.0196-3.5089]	0.7372	[0.2402-13.6339]	0.9339	0.9582
cryptochlorogenic acid	0.0467	[0.0467-0.0467]	0.0467	[0.0467-30.2592]	0.0797	0.1955
cyclo(gly-glu)	0.4902	[0.137-1.4029]	1.4576	[0.1385-17.4513]	**0.0062**	**0.0399**
cyclo(leu-pro)	0.8631	[0.3057-1.5684]	1.5780	[0.8348-3.3178]	**0.0016**	**0.0180**
cyclo(L-phe-D-pro)*	0.7827	[0.2018-2.1343]	0.2018	[0.2018-7.3197]	0.3457	0.5313
cyclo(L-phe-L-pro)	0.7206	[0.1426-1.2121]	1.3444	[0.74-3.5132]	**0.0004**	**0.0069**
cyclo(pro-tyr)	1.1284	[0.3243-2.0727]	0.5416	[0.0719-6.0035]	0.1150	0.2578
cysteine	0.5919	[0.2732-1.4702]	3.1520	[0.1339-15.8029]	**0.0028**	**0.0234**
cysteine s-sulfate	0.0917	[0.0917-1.4858]	0.5429	[0.0917-20.5032]	**0.0141**	0.0670
cystine	0.0107	[0.0107-0.7625]	1.8062	[0.0107-10.6622]	**0.0019**	**0.0197**
cytidine	1.0764	[0.4209-2.156]	0.9288	[0.2431-6.3155]	0.4306	0.5971
cytosine	0.7841	[0.1705-2.2509]	1.0412	[0.0507-18.6288]	0.9337	0.9582
daidzein	1.1912	[0.3435-55.4554]	0.5373	[0.0942-19.8957]	0.0971	0.2254
daidzin	0.2928	[0.2928-1.6971]	0.2928	[0.2928-27.5209]	0.5772	0.7279
decanoylcarnitine	0.7470	[0.4487-2.5299]	1.3312	[0.5576-13.4626]	0.0619	0.1665
dehydrolithocholate	1.5589	[0.1106-7.6316]	0.0963	[0.0155-2.2566]	**0.0002**	**0.0047**
delta-tocopherol	0.3344	[0.1716-5.3081]	0.6727	[0.1716-69.111]	0.5064	0.6616
deoxycarnitine	1.6813	[0.0541-3.2022]	0.3664	[0.0115-4.7919]	**0.0310**	0.1009
deoxycholate	1.1090	[0.3068-2.0284]	0.2000	[0.031-3.1216]	**0.0077**	**0.0452**
dihomo-linoleate (20:2n6)	0.5791	[0.2649-9.4356]	3.1205	[0.088-30.7256]	0.0620	0.1665
dihomo-linolenate (20:3n3 or n6)	0.7544	[0.269-3.8493]	1.6784	[0.042-23.9963]	0.2134	0.3851
dihydrocaffeate	2.5192	[0.1268-26.9725]	0.0012	[0.0012-2.614]	**0.0000**	**0.0026**
dihydroferulic acid	1.8901	[0.1218-49.341]	0.4231	[0.0415-4.4669]	**0.0202**	0.0811
dihydrokaempferol	0.1938	[0.1938-2.7884]	0.2815	[0.1938-3.4983]	0.1917	0.3574
dimethylmalonic acid	1.0350	[0.4145-5.0389]	0.7379	[0.3877-1.5797]	**0.0276**	0.0947
docosadienoate (22:2n6)	0.5311	[0.1628-2.1075]	3.9325	[0.0778-37.4996]	**0.0114**	0.0579
docosahexaenoate (DHA; 22:6n3)	0.5938	[0.0391-2.9426]	1.2485	[0.0152-9.9655]	0.1585	0.3118
docosapentaenoate (n3 DPA; 22:5n3)	0.8682	[0.1141-2.5021]	1.6792	[0.0182-12.578]	0.0971	0.2254
docosapentaenoate (n6 DPA; 22:5n6)	0.4811	[0.0689-3.2645]	2.0835	[0.0689-21.7583]	**0.0121**	0.0610
docosatrienoate (22:3n3)	0.1470	[0.147-1.665]	0.7175	[0.147-21.5294]	**0.0019**	**0.0197**
dodecanedioate	1.0566	[0.3575-19.9232]	0.7331	[0.3575-5.0465]	0.1298	0.2747
D-urobilin	1.3001	[0.0653-3.7205]	0.4155	[0.0078-3.4049]	**0.0202**	0.0811
ectoine	1.1464	[0.3234-5.9625]	0.3206	[0.1067-7.7068]	0.0503	0.1467
eicosapentaenoate (EPA; 20:5n3)	0.9095	[0.1054-2.0784]	1.2148	[0.0363-12.0315]	0.2808	0.4703
eicosenoate (20:1n9 or 11)	0.7824	[0.2421-1.7669]	1.8572	[0.2492-29.0449]	**0.0225**	0.0848
enterodiol	0.0076	[0.0076-15.2125]	0.0076	[0.0076-4.0774]	0.6146	0.7537
enterolactone	1.0797	[0.2382-2.9822]	0.4202	[0.0391-30.3657]	0.2444	0.4265
equol	1.7696	[0.1072-7.7133]	0.2004	[0.0744-5.3247]	**0.0201**	0.0811
equol sulfate	0.0867	[0.0867-9.771]	0.0867	[0.0867-5.1527]	0.9363	0.9595
ergosterol	0.4491	[0.4491-5.4227]	0.4491	[0.4491-4.5891]	0.3992	0.5808

Compound	Healthy Median	Healthy Range	Chronic Enteropathy Median	Chronic Enteropathy Range	*p*-value	*q*-value
ergothioneine	0.1203	[0.1203-1.2495]	1.5131	[0.1203-7.4744]	**0.0020**	**0.0203**
eriodictyol	0.4981	[0.037-10.653]	0.0370	[0.037-0.037]	**0.0001**	**0.0032**
erucate (22:1n9)	0.8662	[0.2359-3.0728]	1.9105	[0.3982-10.5099]	**0.0070**	**0.0418**
erythritol	0.3989	[0.3161-3.179]	0.7876	[0.3161-2.8493]	0.2391	0.4210
erythronate*	1.1314	[0.2299-3.2617]	0.8838	[0.2594-2.6643]	0.6482	0.7729
ethanolamine	0.8655	[0.214-1.7854]	1.6988	[0.2618-13.5924]	**0.0079**	**0.0453**
ethylmalonate	0.9881	[0.4826-2.1593]	1.0467	[0.3339-7.3547]	0.8035	0.8856
famotidine	0.0005	[0.0005-0.0005]	0.0005	[0.0005-2.5878]	0.0797	0.1955
ferulate	2.1360	[0.4241-7.6741]	0.2345	[0.0179-1.338]	**0.0002**	**0.0047**
ferulic acid 4-sulfate	0.0014	[0.0014-15.6197]	0.6019	[0.0014-2.427]	0.0898	0.2162
formiminoglutamate	0.7867	[0.167-3.8827]	3.2184	[0.0384-41.863]	0.2997	0.4805
formononetin	0.0469	[0.0469-3.2028]	0.0469	[0.0469-5.1352]	0.4557	0.6205
fructose	0.8215	[0.3416-1.8289]	1.1211	[0.2879-5.3781]	0.7400	0.8416
fucose	0.4958	[0.0885-5.1006]	2.0993	[0.1529-19.6986]	**0.0161**	0.0702
fucosterol	1.9011	[0.5899-3.413]	0.3822	[0.111-3.6991]	**0.0002**	**0.0055**
fuculose	0.4687	[0.1667-4.6004]	0.9562	[0.1667-26.6826]	0.1785	0.3400
fumarate	0.6252	[0.1852-1.5367]	1.2932	[0.0124-5.9547]	0.1844	0.3447
galactonate	0.5963	[0.1025-5.7109]	5.0929	[0.2516-18.728]	**0.0016**	**0.0180**
galactose	0.9608	[0.1496-1.8067]	1.0000	[0.0318-8.506]	0.4306	0.5971
gallate	0.5614	[0.5614-1.4095]	0.5614	[0.5614-1.1622]	0.3894	0.5686
gamma-aminobutyrate (GABA)	0.9001	[0.2594-35.9794]	2.6432	[0.0411-89.9461]	0.3615	0.5391
gamma-CEHC	1.0398	[0.0305-10.8595]	0.3602	[0.0305-20.1215]	0.1701	0.3276
gamma-glutamylisoleucine*	0.8122	[0.1304-1.7543]	1.9676	[0.1655-9.8498]	**0.0279**	0.0947
gamma-glutamylleucine	0.0343	[0.0343-1.9551]	1.4741	[0.0343-18.2079]	**0.0012**	**0.0147**
gamma-glutamyllysine	0.0281	[0.0281-0.1794]	3.1473	[0.0281-10.7836]	**0.0008**	**0.0104**
gamma-glutamylmethionine	0.3542	[0.1115-1.8904]	0.3860	[0.1115-31.8463]	0.6970	0.8175
gamma-glutamylphenylalanine	0.0613	[0.0368-3.5338]	2.6898	[0.0368-27.628]	**0.0008**	**0.0106**
gamma-glutamylvaline	0.2540	[0.254-0.6744]	0.7013	[0.254-10.7623]	**0.0008**	**0.0104**
gamma-tocopherol	0.8585	[0.1998-5.5337]	1.1157	[0.1998-61.8443]	0.3506	0.5348
gamma-tocotrienol	0.5010	[0.501-1.5017]	0.5010	[0.501-0.501]	**0.0036**	**0.0269**
genistein	1.7193	[0.3711-116.3805]	0.4665	[0.0526-57.0113]	0.0745	0.1904
gentisate	1.3543	[0.2759-2.8249]	0.0918	[0.0321-3.6823]	**0.0048**	**0.0325**
gluconate	0.4531	[0.1682-2.3834]	6.3580	[0.1682-35.3436]	**0.0373**	0.1180
glucose	0.7219	[0.1612-3.7221]	2.0046	[0.1557-54.6435]	**0.0421**	0.1285
glucuronate	0.6926	[0.2505-4.0529]	2.7871	[0.1578-28.0646]	**0.0070**	**0.0418**
glutamate	0.7551	[0.2745-2.1437]	3.7114	[0.3543-12.9027]	**0.0003**	**0.0059**
glutamate, gamma-methyl ester	0.7750	[0.0874-6.1946]	1.0030	[0.0874-7.259]	0.8518	0.9159
glutamine	0.6706	[0.0854-3.028]	1.4101	[0.0103-5.7604]	0.3615	0.5391
glutamine-isoleucine	0.9306	[0.0256-7.0585]	1.0694	[0.0643-4.0537]	0.8682	0.9196
glutamine-leucine	1.6778	[0.0911-4.2214]	0.6344	[0.2356-3.6566]	0.2134	0.3851
glutarate (pentanedioate)	1.0512	[0.4831-9.6505]	0.4954	[0.0507-10.1717]	0.2808	0.4703
glutarylcarnitine (C5)	0.1982	[0.1576-2.1669]	1.3508	[0.1576-8.4783]	**0.0190**	0.0793
glycerate	0.8269	[0.2641-1.6839]	1.0514	[0.3592-3.3561]	0.0745	0.1904
glycerol	1.0368	[0.5434-2.6512]	0.8127	[0.0563-7.6995]	0.4553	0.6205
glycerol 3-phosphate (G3P)	0.6084	[0.2317-3.181]	1.5685	[0.7428-8.6916]	**0.0070**	**0.0418**
glycerophosphoethanolamine	1.0278	[0.1582-36.0789]	0.9722	[0.2461-18.9973]	0.7716	0.8650
glycerophosphoinositol*	1.2784	[0.1855-5.3971]	0.5389	[0.1855-10.1725]	0.6782	0.7979
glycerophosphorylcholine (GPC)	1.0630	[0.3007-38.2926]	0.9491	[0.1138-42.7964]	0.5067	0.6616
glycine	0.5176	[0.1702-1.3632]	2.8586	[0.5056-21.2532]	**0.0001**	**0.0032**
glycitein	1.0139	[0.0165-84.8169]	0.4969	[0.0165-18.8669]	0.3951	0.5758
glycochenodeoxycholate	1.0147	[0.1856-2.0846]	0.2644	[0.1856-279.2771]	0.0654	0.1745
glycocholate	0.5941	[0.0972-2.3384]	1.8127	[0.0972-16.2626]	0.2987	0.4805
glycodeoxycholate	0.7217	[0.068-7.4286]	0.0680	[0.068-25.1657]	**0.0034**	**0.0263**
glycohyocholate	0.3508	[0.1785-17.7669]	0.1785	[0.1785-459.1869]	**0.0217**	0.0848

Compound	Healthy		Chronic Enteropathy			
	Median	Range	Median	Range	*p*-value	*q*-value
glycohyodeoxycholate	0.7772	[0.1837-40.7114]	0.1837	[0.1837-480.5528]	0.0929	0.2222
glycolithocholate	0.4590	[0.0459-2.5831]	0.0459	[0.0459-7.9551]	**0.0151**	0.0699
glycoursodeoxycholate	0.3240	[0.324-5.4671]	0.3240	[0.324-13.5823]	0.3617	0.5391
glycylglycine	0.7026	[0.1931-2.4905]	3.2186	[0.0703-7.4394]	0.1465	0.2981
glycylisoleucine	0.6329	[0.1125-4.2771]	1.0405	[0.007-5.5439]	0.2998	0.4805
glycylleucine	0.6638	[0.1363-3.7619]	1.1022	[0.0563-4.821]	0.4068	0.5810
glycylmethionine	1.0000	[0.1945-4.4256]	0.6116	[0.0624-3.6352]	0.7089	0.8229
glycylphenylalanine	0.9655	[0.2124-3.6308]	1.0324	[0.0899-4.0421]	0.4068	0.5810
glycylproline	0.7459	[0.2382-2.9478]	1.7840	[0.2241-4.4342]	**0.0128**	0.0627
glycylserine	0.7633	[0.1733-3.2348]	1.2659	[0.1176-7.0317]	0.2455	0.4265
glycyltryptophan	0.7082	[0.1282-2.1002]	1.3007	[0.0718-2.9842]	0.1354	0.2811
glycyltyrosine	1.1496	[0.174-5.0155]	0.9137	[0.07-3.0134]	0.9339	0.9582
glycylvaline	0.4630	[0.1151-2.8342]	1.4732	[0.0118-6.7495]	0.0620	0.1665
guanidinoacetate	0.5949	[0.3773-2.9214]	0.3773	[0.3773-1.6302]	0.1167	0.2601
guanine	0.8369	[0.2131-2.7086]	1.3452	[0.0289-13.3948]	0.9669	0.9743
guanosine	1.1604	[0.1224-4.2997]	0.8046	[0.0777-39.1072]	0.2806	0.4703
gulonic acid*	0.1309	[0.0286-0.5437]	8.8565	[0.0286-28.1393]	**0.0003**	**0.0069**
harmane	1.0208	[0.3991-2.3042]	0.9078	[0.1063-28.6321]	0.4067	0.5810
heme	0.1166	[0.1166-0.1166]	0.1166	[0.1166-98.833]	**0.0181**	0.0758
hesperetin	0.0589	[0.0589-3.7745]	0.0589	[0.0589-0.7284]	0.1458	0.2981
hexadecanedioate	1.0214	[0.5147-2.0487]	0.9327	[0.317-3.0223]	0.3837	0.5613
hexanoylcarnitine	0.4685	[0.0963-1.3768]	0.3351	[0.0963-8.0447]	0.8649	0.9196
hippurate	0.1301	[0.1301-1]	0.1301	[0.1301-68.5918]	0.2075	0.3798
histamine	0.9359	[0.0798-379.1795]	0.8375	[0.0798-201.148]	0.4063	0.5810
histidine	0.4237	[0.1779-2.6708]	2.5225	[0.5924-20.0847]	**0.0003**	**0.0059**
histidylisoleucine	1.1035	[0.2729-7.7476]	0.9492	[0.0308-4.2677]	0.4306	0.5971
histidylleucine	1.6475	[0.1268-5.8465]	0.8589	[0.0463-3.875]	0.6482	0.7729
histidylmethionine	0.6762	[0.236-6.8684]	0.2360	[0.236-3.7845]	0.0661	0.1758
histidylphenylalanine	1.0631	[0.2379-5.815]	0.5872	[0.1733-5.0885]	0.1985	0.3659
histidylproline	0.6647	[0.1688-3.2254]	1.2286	[0.1082-4.5712]	0.1585	0.3118
histidyltryptophan	0.1865	[0.1865-2.7243]	0.8736	[0.1865-3.2765]	0.1336	0.2810
homocitrulline	0.9910	[0.2864-2.5545]	1.0451	[0.0385-17.333]	0.6783	0.7979
homoserine	1.2938	[0.2134-4.1918]	0.9081	[0.3028-7.7938]	0.5338	0.6876
hydroquinone sulfate	0.2490	[0.1064-10.9813]	0.1064	[0.1064-7.2782]	0.8246	0.9047
hydroxybutyrylcarnitine*	0.2415	[0.2415-1.6117]	0.4220	[0.2415-524.1505]	0.1258	0.2684
hyocholate	0.8646	[0.2008-7.271]	1.1384	[0.0865-51.0266]	0.5614	0.7115
hyodeoxycholate	2.0594	[0.063-13.5884]	0.5293	[0.0535-6.9848]	0.0619	0.1665
hypoxanthine	1.0274	[0.1663-1.9701]	0.6523	[0.0355-7.0067]	0.4805	0.6423
imidazole lactate	0.9784	[0.0315-2.6191]	1.0216	[0.0343-5.1134]	0.2808	0.4703
imidazole propionate	2.0383	[0.0903-4.2588]	0.2984	[0.0008-3.2014]	**0.0021**	**0.0203**
indole	1.5029	[0.0557-12.0477]	0.2013	[0.0149-31.9668]	0.2132	0.3851
indole-3-carboxylic acid	1.0040	[0.1511-7.8426]	0.4706	[0.1511-4.2025]	0.5577	0.7115
indoleacetate	2.2174	[0.3971-8.0187]	0.3226	[0.0124-3.9607]	**0.0004**	**0.0074**
indoleacetylglutamine	0.0715	[0.0715-7.4572]	0.7234	[0.0715-18.5283]	0.0761	0.1913
indolelactate	0.3349	[0.0095-5.732]	1.6964	[0.0876-27.606]	**0.0465**	0.1370
indolepropionate	1.2305	[0.0046-3.109]	0.0080	[0.0046-2.2127]	**0.0043**	**0.0296**
indolin-2-one	2.5225	[0.1104-206.8175]	0.1584	[0.0456-87.3051]	**0.0062**	**0.0399**
inosine	1.2614	[0.0117-3.1333]	0.0308	[0.0117-4.1163]	**0.0498**	0.1457
inositol 1-phosphate (I1P)	0.5902	[0.5902-2.2349]	0.5902	[0.5902-3.0773]	0.7254	0.8383
isobutyrylcarnitine	1.0182	[0.6647-2.2435]	0.9737	[0.2239-2.5355]	0.5069	0.6616
isobutyrylglycine	0.8090	[0.4577-2.301]	1.3991	[0.1442-14.8308]	0.1249	0.2670
isocaproate	1.1368	[0.3209-12.7417]	0.6891	[0.1247-178.9493]	0.0890	0.2149
isoferulate	1.6912	[0.6858-5.9585]	0.4961	[0.0114-2.1368]	**0.0001**	**0.0033**
isoleucine	0.5781	[0.1843-2.0366]	1.7416	[0.4858-5.5138]	**0.0028**	**0.0234**

Compound	Healthy		Chronic Enteropathy			
	Median	Range	Median	Range	*p*-value	*q*-value
isoleucylalanine	1.1012	[0.2031-4.4824]	0.7407	[0.1253-3.775]	0.4807	0.6423
isoleucylarginine	0.9787	[0.0164-16.8003]	0.3365	[0.0164-36.644]	0.3613	0.5391
isoleucylasparagine	1.9755	[0.228-7.6853]	0.8093	[0.2136-4.0345]	0.1844	0.3447
isoleucylaspartate	1.0343	[0.2662-6.0087]	0.9103	[0.3595-4.7812]	0.6482	0.7729
isoleucylglutamate	1.0370	[0.4558-3.4061]	0.7064	[0.2272-1.8801]	0.3195	0.5032
isoleucylglutamine	1.0084	[0.2143-3.6505]	0.8351	[0.4444-4.5915]	0.6482	0.7729
isoleucylglycine	1.5120	[0.4146-5.0287]	0.7535	[0.0913-3.7164]	0.1585	0.3118
isoleucylisoleucine	1.0392	[0.1752-6.7627]	0.9608	[0.0519-4.233]	0.4807	0.6423
isoleucylleucine	1.6319	[0.1532-5.1315]	0.5812	[0.2297-4.1468]	0.1466	0.2981
isoleucylmethionine	1.3307	[0.1409-5.1803]	0.6464	[0.2048-3.8707]	0.1585	0.3118
isoleucylphenylalanine	1.0124	[0.0534-4.8817]	0.8805	[0.1176-3.7995]	0.7400	0.8416
isoleucylserine	1.3582	[0.4514-4.4033]	0.6987	[0.0729-3.2089]	0.2290	0.4078
isoleucylthreonine	0.9950	[0.3617-5.6412]	1.0050	[0.4191-7.0337]	0.4807	0.6423
isoleucyltyrosine	1.1486	[0.1181-3.4855]	0.5986	[0.1843-3.6997]	0.8357	0.9047
isoleucylvaline	1.0767	[0.2609-6.2654]	0.9233	[0.1502-3.5187]	0.5897	0.7349
isovalerate	1.2972	[0.814-3.3495]	0.2571	[0.051-4.3824]	**0.0032**	**0.0254**
isovalerylcarnitine	0.1571	[0.0344-2.1643]	0.7689	[0.0344-19.8732]	0.1225	0.2670
isovalerylglycine	0.7917	[0.4167-2.3484]	1.6994	[0.1556-21.9836]	0.1711	0.3276
I-urobilinogen	1.3736	[0.0646-3.3286]	0.2769	[0.0103-8.0747]	0.1247	0.2670
kaempferol	0.0565	[0.0565-2.4342]	0.1345	[0.0565-7.2782]	0.2332	0.4134
kynurenate	0.6034	[0.0353-5.5782]	6.1762	[0.0246-149.7087]	0.1466	0.2981
kynurenine	0.9338	[0.2531-1.7414]	1.1637	[0.2392-4.6628]	0.4068	0.5810
lactate	0.4862	[0.073-9.0636]	10.2443	[0.4733-90.2055]	**0.0000**	**0.0026**
lanthionine	0.4694	[0.4377-1.2892]	1.2289	[0.4377-27.3279]	0.0740	0.1904
lathosterol	0.5720	[0.572-1.6202]	0.5720	[0.572-2.2804]	0.3375	0.5248
laurate (12:0)	0.7866	[0.6073-6.558]	1.2449	[0.4754-117.2933]	0.1466	0.2981
leucine	0.4169	[0.1763-1.9169]	1.2891	[0.479-4.5303]	**0.0032**	**0.0254**
leucylalanine	1.0699	[0.114-5.9913]	0.7242	[0.042-4.6552]	0.5897	0.7349
leucylarginine	0.8561	[0.0741-12.3698]	0.7490	[0.0741-29.6941]	0.6179	0.7537
leucylasparagine	1.3524	[0.1645-5.8008]	0.5228	[0.3825-5.1318]	0.5614	0.7115
leucylglutamate	0.9240	[0.2174-6.7874]	1.0207	[0.1875-5.4197]	0.6783	0.7979
leucylglutamine*	1.1214	[0.137-3.5215]	0.8218	[0.1749-2.9412]	0.7400	0.8416
leucylglycine	1.8423	[0.2973-5.397]	0.8515	[0.1027-3.7911]	0.3837	0.5613
leucylisoleucine	1.0105	[0.0253-6.8219]	0.8324	[0.0252-7.4673]	0.6187	0.7537
leucylleucine	1.9392	[0.1151-7.15]	0.8279	[0.2856-7.9803]	0.1249	0.2670
leucylmethionine	1.3395	[0.091-6.7933]	0.4107	[0.0915-7.3713]	0.1985	0.3659
leucylphenylalanine	1.7947	[0.0675-8.2906]	0.5895	[0.0845-8.6767]	0.1249	0.2670
leucylserine	1.0094	[0.182-5.3546]	0.9906	[0.1273-6.2893]	0.9010	0.9379
leucylthreonine	1.0054	[0.1902-6.533]	0.9718	[0.2391-6.9845]	0.9010	0.9379
leucyltryptophan	1.0312	[0.0213-6.1123]	0.7694	[0.0055-11.5711]	0.6482	0.7729
leucyltyrosine	1.6853	[0.0791-6.4241]	0.5889	[0.1591-9.273]	0.1585	0.3118
leu-leu-leu	1.1672	[0.0104-5.4667]	0.8026	[0.0104-2.7716]	0.6333	0.7680
levulinate (4-oxovalerate)	1.3258	[0.5905-2.1895]	0.5392	[0.1772-2.7713]	**0.0037**	**0.0269**
linoleate (18:2n6)	0.9318	[0.3306-2.6735]	1.2443	[0.1265-8.5233]	0.8682	0.9196
linolenate [alpha or gamma; (18:3n3 or 6)]	0.9875	[0.4298-15.8315]	1.0125	[0.1104-24.1062]	1.0000	1.0000
lithocholate	1.6371	[0.3585-2.2675]	0.1353	[0.0004-2.6402]	**0.0114**	0.0579
L-urobilin	0.0618	[0.0618-6.7862]	0.0618	[0.0618-3.971]	0.1016	0.2345
lysine	1.0084	[0.1225-4.0209]	0.9916	[0.3226-7.8489]	0.9669	0.9743
lysylisoleucine	0.9584	[0.1889-8.1558]	1.0416	[0.0136-3.8709]	0.4306	0.5971
lysylleucine	1.1926	[0.1716-5.4699]	0.8108	[0.0667-2.9794]	0.2808	0.4703
lysylmethionine	1.3352	[0.1229-6.2667]	0.1495	[0.1229-2.856]	**0.0069**	**0.0418**
lysylphenylalanine	1.1220	[0.0949-6.6373]	0.5839	[0.0554-2.4102]	**0.0251**	0.0894
lysylvaline	1.0795	[0.1627-7.9987]	0.7947	[0.0873-5.845]	0.3837	0.5613
malate	1.7895	[0.2687-6.7684]	0.6892	[0.2132-24.1658]	0.0745	0.1904

Compound	Healthy		Chronic Enteropathy			
	Median	Range	Median	Range	*p*-value	*q*-value
maleate (*cis*-Butenedioate)	0.7797	[0.4116-4.1214]	1.0050	[0.7139-2.2136]	0.2998	0.4805
malonate (propanedioate)	1.1799	[0.1998-5.2286]	0.6125	[0.4086-2.6032]	0.1354	0.2811
maltol	1.9456	[0.4613-169.3159]	0.7783	[0.122-3.6488]	0.0564	0.1563
maltose	1.1072	[0.1898-3.1553]	0.7753	[0.0223-23.7012]	0.4551	0.6205
maltotriose	0.5475	[0.1601-2.6827]	1.2784	[0.1601-73.3269]	0.0808	0.1975
mannitol	0.4358	[0.4358-1]	0.4358	[0.4358-5.648]	0.6894	0.8098
mannose	1.0322	[0.1633-1.9984]	0.9678	[0.3113-4.3963]	0.5069	0.6616
margarate (17:0)	0.8972	[0.6393-1.9519]	1.0306	[0.5941-4.4826]	0.4306	0.5971
methionine	0.5587	[0.2609-2.2128]	1.5776	[0.3513-6.8466]	**0.0161**	0.0702
methionine sulfone	0.3685	[0.2526-4.6328]	0.2526	[0.2526-137.5423]	0.7929	0.8839
methionine sulfoxide	0.7986	[0.3646-1.7235]	1.0192	[0.3076-7.6077]	0.1844	0.3447
methionylalanine	0.6625	[0.0787-10.9809]	0.2397	[0.0787-14.176]	0.3130	0.4966
methionylglutamate	0.3073	[0.0921-4.3703]	0.0921	[0.0921-3.8855]	0.0989	0.2289
methionylphenylalanine	0.9758	[0.1027-6.2819]	0.9005	[0.1027-15.4082]	0.9339	0.9582
methionylserine	0.3122	[0.3122-1.6737]	0.3122	[0.3122-3.4606]	0.7293	0.8416
methionylvaline	1.1352	[0.0579-12.5163]	0.8648	[0.2681-16.0681]	0.9010	0.9379
methyl indole-3-acetate	1.1436	[0.4862-3.5905]	0.1987	[0.0378-10.5699]	**0.0037**	**0.0269**
methylphosphate	0.6756	[0.1029-4.0833]	2.1897	[0.1029-8.0602]	**0.0170**	0.0733
methylsuccinate	1.2118	[0.3609-4.1725]	0.5098	[0.0052-6.1487]	0.0680	0.1790
mevalonate	0.3868	[0.0472-7.787]	4.2092	[0.0472-43.5754]	**0.0114**	0.0579
mevalonolactone	0.4506	[0.1647-9.4323]	2.9927	[0.0526-38.4472]	**0.0028**	**0.0234**
myo-inositol	0.7745	[0.2621-2.0393]	5.3074	[0.2857-10.8425]	**0.0037**	**0.0269**
myristate (14:0)	0.9998	[0.6655-1.6573]	1.1191	[0.6353-7.2903]	0.4553	0.6205
myristoleate (14:1n5)	1.0535	[0.6644-1.9179]	0.7954	[0.2162-4.8019]	0.3837	0.5613
N(1)-acetylspermine	0.6589	[0.0105-9.1911]	0.6621	[0.0105-28.6884]	0.8679	0.9196
N1-Methyl-2-pyridone-5-carboxamide	0.8981	[0.3304-2.2027]	0.6215	[0.3304-2.7688]	0.5011	0.6616
N1-methylguanosine	0.8149	[0.0386-1.7804]	0.3059	[0.0386-2.8636]	0.6121	0.7537
N2,N2-dimethylguanine	0.5976	[0.1282-1.7342]	4.9153	[0.127-43.9334]	**0.0090**	**0.0487**
N2,N2-dimethylguanosine	0.3739	[0.3739-0.7571]	0.4611	[0.3739-58.8184]	**0.0416**	0.1285
N2-acetyllysine	1.0586	[0.3844-2.3008]	0.9414	[0.0918-5.5017]	0.8357	0.9047
N6-acetyllysine	0.8721	[0.6339-2.4104]	1.7057	[0.3792-8.769]	0.0564	0.1563
N6-carbamoylthreonyladenosine	0.2621	[0.2621-1.6474]	0.2621	[0.2621-55.4916]	0.2492	0.4320
N6-carboxyethyllysine	0.8715	[0.3417-2.5515]	1.3624	[0.0236-2.7891]	0.3837	0.5613
N6-carboxymethyllysine	0.7880	[0.0861-2.5362]	1.1981	[0.065-12.459]	0.2998	0.4805
N6-succinyladenosine	0.2829	[0.2829-3.1924]	0.2829	[0.2829-124.8342]	0.6545	0.7784
N-6-trimethyllysine	0.9628	[0.1098-1.8317]	1.0372	[0.0635-1.9979]	0.3401	0.5248
N-acetyl-1-methylhistidine*	0.9945	[0.4244-2.1494]	1.1791	[0.1765-4.9063]	0.5338	0.6876
N-acetyl-3-methylhistidine*	1.5886	[0.0448-8.0379]	0.2304	[0.0448-1.9325]	**0.0019**	**0.0197**
N-acetylalanine	0.4338	[0.0485-1.6109]	1.8869	[0.5046-6.7191]	**0.0000**	**0.0026**
N-acetylarginine	0.7304	[0.3099-1.8675]	1.2235	[0.4105-5.7925]	**0.0310**	0.1009
N-acetylaspartate (NAA)	0.8856	[0.0724-6.2627]	0.9280	[0.0724-31.0719]	0.5613	0.7115
N-acetyl-aspartyl-glutamate (NAAG)	0.5251	[0.1299-1.7086]	2.4408	[0.1299-32.8893]	0.0581	0.1605
N-acetyl-beta-alanine	1.4453	[0.5939-3.8111]	0.4648	[0.008-3.1774]	**0.0009**	**0.0115**
N-acetyl-cadaverine	0.6898	[0.0797-11.6022]	1.2741	[0.0011-19.9897]	0.2134	0.3851
N-acetylcarnosine	0.6155	[0.0781-6.0314]	1.2371	[0.0781-17.0273]	0.4543	0.6205
N-acetylcysteine	0.7513	[0.0594-1.3467]	2.2476	[0.4351-10.4059]	**0.0004**	**0.0069**
N-acetylgalactosamine	0.8668	[0.215-2.9456]	1.2963	[0.1801-58.9752]	0.1585	0.3118
N-acetylglucosamine	1.4174	[0.2408-6.8372]	0.7807	[0.0906-37.8354]	0.5338	0.6876
N-acetylglucosamine 6-phosphate	0.4755	[0.4755-0.4755]	0.4755	[0.4755-11.6402]	0.1644	0.3210
N-acetylglucosamine 6-sulfate	0.0175	[0.0175-4.357]	0.4051	[0.0175-26.3133]	**0.0040**	**0.0281**
N-acetylglutamate	0.9252	[0.3512-2.2829]	1.0891	[0.297-9.4748]	0.2808	0.4703
N-acetylglutamine	0.7263	[0.2533-2.5408]	1.0294	[0.3521-10.5115]	0.0564	0.1563
N-acetylglycine	0.5585	[0.047-4.7485]	1.4340	[0.1216-11.9025]	**0.0344**	0.1092
N-acetylhistidine	0.5093	[0.0873-3.3933]	1.5794	[0.6529-5.4098]	**0.0090**	**0.0487**

Compound	Healthy		Chronic Enteropathy			
	Median	Range	Median	Range	*p*-value	*q*-value
N-acetylisoleucine	0.6130	[0.0838-4.6842]	1.4465	[0.0336-5.1139]	0.2134	0.3851
N-acetylleucine	0.8454	[0.2084-4.9104]	1.4998	[0.1126-4.519]	0.6187	0.7537
N-acetylmannosamine	0.4952	[0.1401-2.0868]	1.2288	[0.1401-17.7335]	0.0774	0.1941
N-acetylmethionine	0.3509	[0.1429-1.8227]	3.6530	[0.7346-16.4718]	**0.0000**	**0.0020**
N-acetylmethionine sulfoxide	0.3287	[0.3287-0.3287]	0.5189	[0.3287-9.2601]	**0.0001**	**0.0032**
N-acetylmuramate	1.4119	[0.3958-6.0812]	0.4223	[0.0532-2.9808]	**0.0021**	**0.0203**
N-acetylneuraminate	0.8988	[0.2108-6.1778]	1.6429	[0.2444-21.1627]	0.1585	0.3118
N-acetylphenylalanine	1.5671	[0.1504-5.0197]	0.9714	[0.1327-6.0393]	0.4306	0.5971
N-acetylproline	1.3076	[0.7151-3.2558]	0.5273	[0.1873-4.5547]	**0.0128**	0.0627
N-acetylputrescine	0.8990	[0.2833-4.0944]	1.1010	[0.1796-8.0225]	0.6187	0.7537
N-acetylserine	0.2241	[0.1285-2.0652]	1.9534	[0.3055-15.6268]	**0.0004**	**0.0074**
N-acetylsphingosine	0.9746	[0.0173-15.3395]	0.6240	[0.0157-142.5131]	0.5067	0.6616
N-acetyltaurine	1.3918	[0.0132-3.1677]	0.8436	[0.0403-4.1067]	0.7715	0.8650
N-acetylthreonine	0.2316	[0.0773-2.3719]	2.8786	[0.3868-6.6983]	**0.0001**	**0.0033**
N-acetyltryptophan	0.8367	[0.0515-3.4033]	1.4351	[0.459-3.446]	0.0620	0.1665
N-acetyltyrosine	1.2193	[0.4393-3.7936]	0.5510	[0.1352-2.2527]	**0.0181**	0.0758
N-acetylvaline	0.7026	[0.1067-2.4609]	1.0214	[0.0944-2.9926]	0.2455	0.4265
N-alpha-acetylornithine	1.3724	[0.517-5.8561]	0.3968	[0.0461-2.6473]	**0.0008**	**0.0104**
naringenin	0.9020	[0.2039-32.9711]	1.0980	[0.2455-13.1332]	0.7716	0.8650
N-carbamoylaspartate	0.2440	[0.244-2.0314]	0.3987	[0.244-104.9065]	0.3110	0.4945
N-delta-acetylornithine	0.6438	[0.257-1.8602]	3.5042	[0.0527-31.8975]	**0.0161**	0.0702
N-formylmethionine	0.7446	[0.2631-1.542]	2.6363	[0.4518-7.3884]	**0.0012**	**0.0147**
N-glycolylneuraminate	1.0550	[0.0632-8.7076]	0.8406	[0.0632-15.7136]	0.7557	0.8557
nicotianamine	0.9708	[0.0024-18.6832]	0.5751	[0.0024-21.8232]	0.4296	0.5971
nicotinamide	0.8978	[0.0532-8.6049]	1.2054	[0.0532-12.2132]	0.3835	0.5613
nicotinamide riboside	0.5659	[0.0793-38.1636]	1.0372	[0.0793-3.0988]	0.5896	0.7349
nicotinate	1.3413	[0.4594-2.3664]	0.8065	[0.0439-1.9499]	**0.0161**	0.0702
nicotinate ribonucleoside	1.5381	[0.0566-39.1645]	0.0698	[0.0566-1.213]	**0.0232**	0.0869
N-linoleoylglycine	0.6803	[0.014-3.1316]	1.5579	[0.014-15.2812]	0.1329	0.2805
N-methyl proline	0.8112	[0.1258-8.4904]	0.6071	[0.1258-1.9264]	0.1296	0.2747
N-methylhydantoin	1.9446	[0.1037-20.325]	0.8849	[0.1005-19.2922]	0.5069	0.6616
N-oleoyltaurine	0.2758	[0.0341-1.4989]	1.0549	[0.0341-28.0029]	**0.0180**	0.0758
norvaline	1.0000	[0.0428-7.9614]	0.8858	[0.0428-8.0614]	0.7555	0.8557
N-palmitoyl glycine	0.5228	[0.2135-3.2187]	1.8104	[0.0396-6.5977]	**0.0251**	0.0894
N-palmitoyl-sphingosine*	0.6692	[0.2844-1.147]	1.3303	[0.2844-4.1936]	**0.0327**	0.1057
N-palmitoyltaurine	0.5120	[0.1012-2.1372]	1.2683	[0.1012-5.6276]	0.0507	0.1472
N-propionylalanine	1.5742	[0.3046-5.0823]	0.2766	[0.0389-5.8376]	**0.0032**	**0.0254**
N-propionylmethionine	1.0713	[0.2505-11.3137]	0.3414	[0.1176-6.3423]	0.0617	0.1665
N-stearoyltaurine	0.8310	[0.1628-2.3516]	1.4454	[0.1628-5.0012]	0.3090	0.4930
O-acetylhomoserine	0.8254	[0.1321-3.368]	1.8185	[0.0943-14.0272]	0.5614	0.7115
O-acetylserine	0.1962	[0.1962-1.4774]	0.3300	[0.1962-2.282]	0.1051	0.2391
oleanolate	0.8285	[0.0399-6.1293]	0.0827	[0.0399-10.863]	0.2391	0.4210
oleate (18:1n9)	0.9748	[0.4464-2.0824]	2.0593	[0.0577-7.5303]	0.5897	0.7349
oleic ethanolamide	0.7977	[0.2461-2.9401]	1.9369	[0.0021-5.2536]	0.1150	0.2578
oleoylcarnitine	0.2725	[0.2725-1.62]	0.2725	[0.2725-12.4497]	0.8587	0.9196
oleoyl-linoleoyl-glycerol	1.3578	[0.1603-3.5371]	0.1110	[0.0169-6.896]	**0.0225**	0.0848
oleoyl-linoleoyl-glycerophosphocholine (1)*	0.2789	[0.2789-1.2705]	0.2789	[0.2789-7.8669]	0.5331	0.6876
oleoyl-linoleoyl-glycerophosphocholine (2)*	0.4134	[0.4134-1]	0.4134	[0.4134-9.4138]	0.9162	0.9525
O-methylcatechol sulfate	0.4697	[0.4697-4.4151]	0.4697	[0.4697-1.4407]	0.3616	0.5391
ornithine	0.3192	[0.049-4.9304]	1.9113	[0.1833-13.0951]	**0.0055**	**0.0364**
orotate	0.4025	[0.0629-2.1987]	2.7536	[0.0316-34.3761]	**0.0048**	**0.0325**
orotidine	0.0136	[0.0136-8.232]	0.5922	[0.0136-1.7869]	**0.0024**	**0.0217**
O-sulfo-L-tyrosine	0.0558	[0.0558-1.7598]	0.7690	[0.0558-9.5363]	**0.0107**	0.0562
o-Tyrosine	0.9317	[0.3473-1.6902]	1.2437	[0.1527-5.5469]	0.0971	0.2254

Compound	Healthy		Chronic Enteropathy			
	Median	Range	Median	Range	*p*-value	*q*-value
palmitate (16:0)	0.8738	[0.6445-1.4488]	1.1657	[0.6272-5.5555]	0.0564	0.1563
palmitoleate (16:1n7)	0.7638	[0.2776-1.9332]	1.2307	[0.1351-23.6481]	0.4306	0.5971
palmitoyl ethanolamide	0.6569	[0.2707-2.5249]	2.2235	[0.0162-4.8556]	**0.0310**	0.1009
palmitoyl sphingomyelin	0.2987	[0.1834-1.5733]	1.3561	[0.1834-6.355]	**0.0107**	0.0562
palmitoyl-arachidonoyl-glycerophosphocholine (1)*	0.3472	[0.3472-1.1809]	0.3472	[0.3472-7.603]	0.5331	0.6876
palmitoyl-arachidonoyl-glycerophosphocholine (2)*	0.2188	[0.2188-1.4614]	0.2188	[0.2188-7.2173]	0.1249	0.2670
palmitoylcarnitine	0.1719	[0.1144-5.5893]	0.5949	[0.1144-10.569]	0.4110	0.5860
palmitoyl-linoleoyl-glycerophosphocholine (1)*	0.4384	[0.4384-1.2896]	0.4384	[0.4384-33.038]	0.7797	0.8729
palmitoyl-linoleoyl-glycerophosphocholine (2)*	0.4885	[0.4885-1.5857]	0.4885	[0.4885-30.9862]	0.3416	0.5261
palmitoyl-oleoyl-glycerophosphocholine (1)*	0.3145	[0.3145-1.1449]	0.3145	[0.3145-30.1359]	0.5736	0.7258
p-aminobenzoate (PABA)	1.9048	[0.8011-4.3147]	0.0673	[0.0198-1.9903]	**0.0000**	**0.0017**
pantothenate	1.5497	[0.3013-6.0816]	0.6014	[0.0994-2.2786]	**0.0279**	0.0947
p-coumaroylserotonin	0.2771	[0.0537-33.7006]	0.0537	[0.0537-4.0689]	0.2677	0.4591
p-cresol sulfate	0.3499	[0.3499-1]	0.3499	[0.3499-233.6838]	0.0750	0.1908
pentadecanoate (15:0)	0.9112	[0.7594-2.1785]	1.0055	[0.48-2.64]	0.6783	0.7979
phenethylamine	0.6548	[0.0883-8.9461]	1.3102	[0.0094-7.7202]	0.3615	0.5391
phenol sulfate	0.4352	[0.0348-5.3274]	0.0348	[0.0348-7.171]	0.2509	0.4340
phenylacetate	1.3037	[0.2174-5.3579]	0.5826	[0.0247-13.7593]	0.1349	0.2811
phenylacetylglutamine	0.1065	[0.0327-8.4714]	1.0521	[0.0327-18.5244]	0.0528	0.1512
phenylacetylglycine	0.4816	[0.0295-1.6398]	1.3304	[0.0295-23.6781]	**0.0259**	0.0918
phenylalanine	0.5339	[0.1921-2.0425]	1.4257	[0.3072-4.2043]	**0.0310**	0.1009
phenylalanylalanine	0.9739	[0.1111-6.7027]	1.0261	[0.1951-6.1052]	0.8357	0.9047
phenylalanylarginine	0.9345	[0.1488-6.7595]	0.9617	[0.1488-32.3949]	0.7872	0.8788
phenylalanylaspartate	0.6665	[0.1661-6.3828]	1.0012	[0.2662-5.4103]	0.5069	0.6616
phenylalanylglutamate	0.5198	[0.1405-5.5148]	1.2352	[0.0283-4.0054]	0.6187	0.7537
phenylalanylglycine	1.2705	[0.1302-4.3107]	0.9490	[0.2299-3.2324]	0.8682	0.9196
phenylalanylhistidine	0.4872	[0.1342-4.2065]	0.2142	[0.1342-7.1927]	0.7620	0.8604
phenylalanylisoleucine	1.3217	[0.0288-7.3781]	0.7816	[0.0159-6.5117]	0.1711	0.3276
phenylalanylleucine	1.7420	[0.0093-8.5977]	0.8275	[0.1548-10.3683]	0.6187	0.7537
phenylalanylmethionine	1.2140	[0.0078-8.8769]	0.5588	[0.0078-9.8138]	0.3506	0.5348
phenylalanylphenylalanine	0.6430	[0.0636-4.8454]	0.8098	[0.0636-5.6553]	1.0000	1.0000
phenylalanylproline	0.6644	[0.1628-2.9365]	1.6147	[0.4955-4.8604]	**0.0202**	0.0811
phenylalanylserine	1.0212	[0.3605-4.198]	0.9327	[0.3684-4.0344]	0.9669	0.9743
phenylalanyltryptophan	0.8775	[0.1285-8.9087]	0.5133	[0.1285-12.491]	0.7376	0.8416
phenylalanyltyrosine	1.8015	[0.0223-7.8066]	0.7619	[0.0223-11.478]	0.2371	0.4193
phenylalanylvaline	1.0759	[0.1015-5.7194]	0.8475	[0.2528-5.0676]	0.7400	0.8416
phenyllactate (PLA)	0.3812	[0.0667-12.3408]	2.7132	[0.2201-19.4645]	**0.0101**	0.0539
phenylpyruvate	1.0228	[0.1113-4.947]	0.6468	[0.0005-10.1771]	0.8682	0.9196
phosphate	1.0724	[0.627-2.868]	0.7021	[0.1805-12.4677]	0.1985	0.3659
phytosphingosine	1.1139	[0.0501-6.5097]	0.7563	[0.0561-37.6992]	0.9669	0.9743
picolinate	1.1109	[0.2772-5.4655]	0.8785	[0.1903-1.5648]	**0.0310**	0.1009
pimelate (heptanedioate)	2.3634	[0.3449-4.9568]	0.5551	[0.1773-2.3047]	**0.0003**	**0.0065**
pinitol	0.2871	[0.2871-40.7924]	0.2871	[0.2871-2.1382]	0.1289	0.2741
pipecolate	1.1938	[0.5257-2.0873]	0.4045	[0.0386-1.7485]	**0.0009**	**0.0115**
piperidine	1.0000	[0.151-5.6826]	0.6844	[0.0111-21.027]	0.6783	0.7979
piperine	0.1011	[0.1011-4.7398]	0.1011	[0.1011-1.9148]	0.9527	0.9724
pro-hydroxy-pro	0.4754	[0.0557-4.6209]	2.0537	[0.1134-189.6809]	**0.0070**	**0.0418**
proline	0.8310	[0.3493-2.092]	1.9242	[0.4733-15.9081]	**0.0128**	0.0627
prolylalanine	0.7082	[0.2686-7.3193]	1.8303	[0.5473-16.7984]	**0.0070**	**0.0418**
prolylglutamine	0.3993	[0.202-14.4046]	1.7645	[0.202-8.5347]	**0.0220**	0.0848
prolylphenylalanine	0.7570	[0.3429-1.8096]	1.9169	[0.523-4.5023]	**0.0062**	**0.0399**
prolylproline	0.6595	[0.3692-1.4203]	3.2255	[0.6759-108.767]	**0.0003**	**0.0065**
prolyltyrosine	0.5422	[0.1614-1.422]	2.3739	[0.3817-7.1006]	**0.0005**	**0.0076**
prolylvaline	0.6540	[0.1721-1.2787]	3.0258	[0.4597-24.2581]	**0.0005**	**0.0076**

Compound	Healthy		Chronic Enteropathy			
	Median	Range	Median	Range	*p*-value	*q*-value
propionylcarnitine	1.0526	[0.05-3.9265]	0.4648	[0.05-7.7325]	0.3090	0.4930
propionylglycine	1.0636	[0.0878-8.2795]	0.9006	[0.0339-8.935]	0.4068	0.5810
pseudouridine	1.4586	[0.0701-3.1938]	0.7967	[0.0394-3.8934]	0.2455	0.4265
pterin	1.5053	[0.4021-3.0586]	0.2751	[0.0301-4.9594]	**0.0021**	**0.0203**
putrescine	0.8324	[0.0288-12.5031]	2.0441	[0.0288-14.4983]	0.3095	0.4930
pyridoxal	1.2049	[0.3797-2.7078]	0.4337	[0.0213-1.8149]	**0.0128**	0.0627
pyridoxate	1.2419	[0.7619-1.8982]	0.2778	[0.0157-1.6155]	**0.0001**	**0.0043**
pyridoxine (Vitamin B6)	0.5446	[0.0781-7.4113]	1.0378	[0.0781-4.7373]	0.2995	0.4805
pyroglutamine*	1.1145	[0.0422-7.5981]	0.4273	[0.0616-4.4217]	0.4553	0.6205
pyroglutamylglycine	0.6904	[0.1929-2.0462]	1.8585	[0.6691-12.4286]	**0.0007**	**0.0098**
pyroglutamylvaline	0.8788	[0.2306-1.9954]	2.5757	[0.4433-10.6917]	0.0971	0.2254
pyruvate	0.1485	[0.0248-8.1992]	1.5872	[0.0039-8.3821]	**0.0202**	0.0811
quinate	0.8080	[0.3159-3.7971]	1.2756	[0.0628-66.104]	0.5897	0.7349
quinolinate	0.6781	[0.1179-3.1273]	0.7877	[0.1179-22.9225]	0.4920	0.6552
rhamnose	0.5895	[0.3459-7.8159]	0.9114	[0.3459-4.9648]	0.8514	0.9159
ribitol	0.4744	[0.4744-1.412]	0.4744	[0.4744-2.0834]	0.8855	0.9367
riboflavin (Vitamin B2)	2.5792	[0.2572-7.661]	0.3963	[0.0592-5.125]	**0.0251**	0.0894
ribonate	0.8504	[0.1249-2.5194]	1.6296	[0.1902-5.9166]	**0.0225**	0.0848
ribose	1.2090	[0.3361-2.8198]	0.5586	[0.0318-4.7704]	**0.0181**	0.0758
ribulose	1.2708	[0.0608-5.6768]	0.6469	[0.0608-8.2404]	0.2778	0.4703
R-mevalonate 5-diphosphate	0.2610	[0.1037-3.1183]	0.9092	[0.1037-70.3439]	0.1735	0.3314
S-(3-hydroxypropyl)mercapturic acid (HPMA)	0.1304	[0.1304-9.2628]	1.0833	[0.1304-15.2403]	**0.0037**	**0.0270**
saccharopine	0.2718	[0.2718-1.1167]	0.5019	[0.2718-5.9352]	**0.0041**	**0.0291**
S-adenosylmethionine (SAM)	0.0779	[0.0779-1.4994]	0.2053	[0.0779-4.8184]	**0.0490**	0.1439
salicylate	1.7308	[0.6189-3.9826]	0.3478	[0.1832-1.3077]	**0.0000**	**0.0016**
sebacate (decanedioate)	1.8117	[0.7936-8.6909]	0.8071	[0.2274-4.2893]	**0.0021**	**0.0203**
secoisolariciresinol	0.2646	[0.0762-231.7039]	0.0762	[0.0762-41.3022]	0.4310	0.5971
secoisolariciresinol diglucoside	0.0395	[0.0395-14.8732]	0.0395	[0.0395-48.4019]	0.2184	0.3933
serine	0.6361	[0.268-2.3248]	1.6584	[0.5586-6.4986]	**0.0161**	0.0702
serotonin (5HT)	1.0000	[0.3561-3.1485]	0.9156	[0.0986-3.3409]	0.8357	0.9047
serylisoleucine	1.0738	[0.1788-7.8245]	0.9806	[0.0509-4.8537]	0.7089	0.8229
serylleucine	1.1194	[0.1626-4.808]	0.8806	[0.2988-3.8626]	0.8357	0.9047
serylmethionine	1.1171	[0.1708-4.6912]	0.7480	[0.0957-6.0954]	0.3615	0.5391
serylphenyalanine	0.8351	[0.089-4.4473]	1.1649	[0.073-4.974]	0.8357	0.9047
serylproline	0.4696	[0.0625-2.0917]	1.1419	[0.2442-5.9571]	**0.0202**	0.0811
seryltyrosine	0.8363	[0.0668-3.5505]	1.0391	[0.0486-3.9364]	0.9339	0.9582
serylvaline	0.9201	[0.275-7.5363]	1.0799	[0.0432-4.6082]	0.6482	0.7729
sinapate	1.3864	[0.3205-6.7161]	0.3201	[0.0252-2.0517]	**0.0160**	0.0702
sitostanol	1.5717	[0.4157-2.3312]	0.1008	[0.0963-0.5215]	**0.0000**	**0.0011**
solanidine	0.0350	[0.035-2.4356]	0.0350	[0.035-839.7977]	0.1028	0.2367
spermidine	1.7785	[0.0132-6.4075]	0.5448	[0.0245-15.963]	0.0745	0.1904
spermine	1.0000	[0.0928-10.5148]	0.2486	[0.0928-220.103]	0.7376	0.8416
sphinganine	0.5691	[0.0145-3.8369]	1.8859	[0.0126-7.4785]	0.1249	0.2670
sphingosine	0.5914	[0.0184-3.8582]	2.4597	[0.0088-10.4542]	0.2290	0.4078
stachydrine	1.8420	[0.0392-3.9074]	0.2314	[0.0082-4.5176]	0.0564	0.1563
stearate (18:0)	0.8805	[0.6107-1.1521]	1.3202	[0.6258-2.9426]	**0.0025**	**0.0217**
stearidonate (18:4n3)	0.9717	[0.1105-5.1855]	1.4701	[0.042-33.8738]	0.8035	0.8856
stearoyl ethanolamide	0.7504	[0.239-4.7974]	1.6622	[0.2024-24.373]	0.1354	0.2811
stearoyl-arachidonoyl-glycerophosphocholine (1)*	0.4470	[0.447-0.447]	0.4470	[0.447-1.553]	0.3506	0.5348
stearoyl-arachidonoyl-glycerophosphocholine (2)*	0.6880	[0.688-0.688]	0.6880	[0.688-1.312]	0.3506	0.5348
stearoylcarnitine	0.1034	[0.1034-3.6945]	0.9726	[0.1034-7.5588]	0.0761	0.1913
suberate (octanedioate)	1.3504	[0.6417-3.3989]	0.8038	[0.4233-1.947]	**0.0090**	**0.0487**
succinate	0.1246	[0.0415-4.7919]	1.7903	[0.4471-10.7129]	**0.0079**	**0.0453**
succinimide	0.9397	[0.4961-1.8106]	1.1551	[0.7528-5.8195]	0.1150	0.2578

Compound	Healthy		Chronic Enteropathy			
	Median	**Range**	**Median**	**Range**	***p*-value**	***q*-value**
succinylcarnitine	0.8544	[0.0117-6.6029]	1.0745	[0.0329-23.3082]	0.3401	0.5248
sulfate*	0.9941	[0.5677-2.5718]	1.0059	[0.0032-7.5965]	0.6482	0.7729
symmetric dimethylarginine (SDMA)	0.4706	[0.0333-3.61]	0.9660	[0.0333-4.6147]	0.5862	0.7349
syringic acid	2.2895	[0.4455-66.7552]	0.2637	[0.0763-1.4336]	**0.0000**	**0.0018**
taurine	0.7111	[0.0609-3.6556]	1.0841	[0.2994-5.8539]	0.1249	0.2670
tauro-alpha-muricholate	0.0263	[0.0263-6.9685]	1.1150	[0.0263-20.2105]	**0.0216**	0.0848
tauro-beta-muricholate	0.7756	[0.072-17.6249]	0.4522	[0.072-21.957]	0.8505	0.9159
taurochenodeoxycholate	0.5053	[0.0839-8.3797]	1.0000	[0.0839-80.9337]	0.8518	0.9159
taurocholate	1.3417	[0.247-13.0527]	0.9403	[0.0556-15.285]	0.2998	0.4805
taurodeoxycholate	1.4618	[0.2589-26.1331]	0.5325	[0.0286-20.4489]	0.1985	0.3659
taurohyodeoxycholic acid	0.7659	[0.0276-13.2326]	0.6211	[0.0276-23.6901]	0.4510	0.6205
taurolithocholate	0.6622	[0.0157-10.4824]	0.1251	[0.0157-33.1628]	0.5577	0.7115
tauroursodeoxycholate	0.1261	[0.0367-8.255]	1.0025	[0.0367-71.4848]	**0.0436**	0.1322
thiamin (Vitamin B1)	1.5225	[0.668-5.1574]	0.0932	[0.0063-6.2646]	**0.0008**	**0.0104**
threitol	0.9548	[0.3853-2.2913]	0.8921	[0.3853-3.5623]	0.9337	0.9582
threonate	1.1130	[0.31-3.4249]	0.7446	[0.1526-3.4797]	0.2290	0.4078
threonine	0.6360	[0.3581-2.1626]	1.6459	[0.3514-9.0978]	**0.0144**	0.0670
threonylalanine	1.0237	[0.2757-4.5588]	0.8852	[0.1425-4.876]	0.6482	0.7729
threonylarginine	1.1587	[0.3798-3.9215]	0.2419	[0.0435-5.2722]	**0.0032**	**0.0254**
threonylglutamate	0.7160	[0.1706-4.8473]	1.3970	[0.3686-2.4215]	0.1585	0.3118
threonylisoleucine	1.3195	[0.2382-6.4691]	0.9655	[0.0273-3.2296]	0.2628	0.4515
threonylleucine	1.1307	[0.12-4.7641]	0.6531	[0.3014-4.4628]	0.2808	0.4703
threonylmethionine	1.4567	[0.0493-5.3704]	0.5121	[0.0826-4.3955]	0.0564	0.1563
threonylphenylalanine	1.7825	[0.136-4.9575]	0.4680	[0.1889-4.3914]	0.1711	0.3276
threonylproline	0.4778	[0.1063-3.3413]	1.8665	[0.4644-6.8027]	**0.0465**	0.1370
threonylvaline	1.1681	[0.2944-6.6518]	0.9873	[0.0633-5.5623]	0.8357	0.9047
thymidine	1.9487	[0.5523-3.6203]	0.5942	[0.0316-3.4646]	**0.0225**	0.0848
thymine	1.0605	[0.203-1.8905]	0.6005	[0.1459-5.0098]	0.1249	0.2670
trans-4-hydroxyproline	0.9616	[0.148-2.1927]	1.3823	[0.0517-10.3233]	0.5069	0.6616
trans-urocanate	0.9326	[0.2668-8.6345]	1.2439	[0.0924-4.8684]	0.8357	0.9047
trigonelline (N'-methylnicotinate)	1.0112	[0.534-1.8551]	0.8812	[0.0987-4.4939]	0.9010	0.9379
tryptamine	1.0000	[0.1618-3.0142]	0.8686	[0.0003-9.7774]	0.7715	0.8650
tryptophan	0.4425	[0.0862-2.5983]	1.3262	[0.0897-4.443]	0.0564	0.1563
tryptophan betaine	0.0234	[0.0234-7.6442]	0.0234	[0.0234-2.9842]	**0.0289**	0.0977
tryptophylalanine	0.9884	[0.0667-5.7616]	0.7126	[0.0667-8.518]	0.7086	0.8229
tryptophylasparagine	1.1608	[0.046-4.8279]	0.7861	[0.046-9.588]	1.0000	1.0000
tryptophylglutamate	1.0225	[0.0663-4.5107]	0.8325	[0.0663-3.7091]	0.6333	0.7680
tryptophylglycine	0.8059	[0.0698-6.4736]	1.0143	[0.1256-4.7842]	0.6482	0.7729
tryptophylisoleucine	1.0000	[0.0317-10.0961]	0.9629	[0.0317-19.7158]	0.6183	0.7537
tryptophylleucine	1.1263	[0.0689-4.5726]	0.6669	[0.106-7.3649]	1.0000	1.0000
tryptophylphenylalanine	0.8741	[0.0825-3.0225]	0.4682	[0.0825-4.0338]	0.8991	0.9379
tryptophylproline	0.3306	[0.0494-2.1671]	1.3504	[0.3355-10.0309]	**0.0048**	**0.0325**
tryptophylvaline	0.7843	[0.0175-4.3806]	1.1528	[0.0175-7.0999]	1.0000	1.0000
tyramine	0.4440	[0.1364-17.5679]	11.9819	[0.0128-30.528]	**0.0014**	**0.0165**
tyrosine	1.2134	[0.3966-4.9352]	0.6149	[0.1736-5.7459]	0.0680	0.1790
tyrosol	1.0025	[0.3722-6.2362]	0.9790	[0.1503-4.4203]	0.4553	0.6205
tyrosylalanine	0.9462	[0.0586-5.7727]	1.0122	[0.1629-8.0887]	0.4807	0.6423
tyrosylarginine	0.7857	[0.2878-5.0722]	0.5556	[0.2878-30.2616]	0.8179	0.9002
tyrosylglutamate	0.6056	[0.0874-4.0663]	1.1150	[0.0783-4.7509]	0.2998	0.4805
tyrosylglutamine	0.9740	[0.0303-4.1567]	1.0000	[0.0303-5.2093]	0.6632	0.7872
tyrosylglycine	1.0169	[0.1466-2.6642]	0.7521	[0.3542-3.5576]	0.8035	0.8856
tyrosylisoleucine	1.1238	[0.1154-4.326]	0.8143	[0.0874-4.5415]	0.9669	0.9743
tyrosylleucine	1.0555	[0.0445-5.8718]	0.8997	[0.0721-7.6498]	0.7400	0.8416
tyrosyllysine	1.4846	[0.0381-25.6346]	0.6924	[0.0381-38.5227]	0.4057	0.5810

Compound	Healthy Median	Healthy Range	Chronic Enteropathy Median	Chronic Enteropathy Range	*p*-value	*q*-value
tyrosylphenylalanine	0.2831	[0.0318-5.3619]	0.1854	[0.0318-8.3315]	0.7620	0.8604
tyrosyltyrosine	0.9059	[0.0732-5.4201]	0.7157	[0.0732-11.6181]	0.9502	0.9724
tyrosylvaline	1.1316	[0.108-5.6607]	0.8476	[0.3471-5.1217]	0.9669	0.9743
undecanedioate	1.8052	[0.8091-3.0282]	0.5537	[0.2821-2.2488]	**0.0002**	**0.0050**
uracil	1.5127	[0.2511-4.4562]	0.5772	[0.0907-4.8754]	**0.0421**	0.1285
urate	1.1064	[0.3069-3.8631]	0.7644	[0.1605-19.5307]	0.5338	0.6876
uridine	1.4232	[0.2623-3.4162]	0.2316	[0.0451-5.9544]	**0.0310**	0.1009
ursodeoxycholate	2.1302	[0.4259-7.3884]	0.5555	[0.0097-7.376]	**0.0279**	0.0947
valerate	1.1264	[0.4982-4.3892]	0.1348	[0.0501-3.522]	**0.0025**	**0.0217**
valine	0.4427	[0.1662-1.8432]	1.7055	[0.3998-6.5297]	**0.0028**	**0.0234**
val-val-val	0.3744	[0.0071-3.9804]	1.0569	[0.0071-4.7791]	0.1583	0.3118
valylalanine	1.0480	[0.3243-4.0153]	0.9695	[0.1738-4.7968]	0.9010	0.9379
valylarginine	0.9471	[0.122-12.5435]	0.2081	[0.122-16.2534]	0.2072	0.3798
valylasparagine	1.8367	[0.0482-7.2386]	0.7536	[0.126-2.9738]	0.2998	0.4805
valylaspartate	0.7566	[0.2051-4.7998]	1.1461	[0.3955-3.146]	0.2454	0.4265
valylglutamate	1.0157	[0.2464-2.399]	0.9843	[0.2725-2.5639]	0.5897	0.7349
valylglutamine	0.6735	[0.1525-2.3502]	1.0667	[0.2987-3.2481]	0.3401	0.5248
valylglycine	1.1147	[0.3569-2.8736]	0.8853	[0.2567-3.7152]	0.9339	0.9582
valylhistidine	0.4528	[0.0235-2.9274]	1.0910	[0.0235-10.2651]	0.1701	0.3276
valylisoleucine	1.1198	[0.1326-6.8543]	0.8119	[0.047-4.1109]	0.5614	0.7115
valylleucine	1.2268	[0.1639-4.5565]	0.7119	[0.2188-3.3866]	0.2290	0.4078
valyllysine	1.3923	[0.0875-7.3626]	0.7605	[0.0875-11.485]	0.2540	0.4383
valylmethionine	1.3388	[0.1484-3.7028]	0.8947	[0.1219-4.6306]	0.3401	0.5248
valylphenylalanine	1.1689	[0.0984-4.1062]	0.7379	[0.2-4.994]	0.8035	0.8856
valylserine	0.9016	[0.2215-3.3537]	1.0984	[0.1228-3.9326]	0.8035	0.8856
valylthreonine	0.8029	[0.1869-4.2412]	1.3239	[0.3871-8.4219]	0.0971	0.2254
valyltryptophan	1.4044	[0.0875-4.1927]	0.8130	[0.3676-4.485]	0.8035	0.8856
valyltyrosine	1.1025	[0.0229-5.0358]	0.6055	[0.0229-6.5121]	0.4805	0.6423
valylvaline	1.0557	[0.307-6.458]	0.9230	[0.1713-3.9734]	0.6187	0.7537
vanillate	1.3059	[0.3711-29.6286]	0.8183	[0.0808-2.2396]	0.0564	0.1563
xanthine	0.9482	[0.1918-2.3439]	1.0382	[0.0238-4.5447]	0.9669	0.9743
xanthosine	1.0384	[0.0172-7.2495]	0.0172	[0.0172-3.5994]	**0.0153**	0.0702
xanthurenate	0.7430	[0.2069-11.0592]	5.4403	[0.1734-203.8674]	0.1349	0.2811
xylitol	0.0734	[0.0734-2.2296]	0.7697	[0.0734-2.9365]	0.0511	0.1472
xylonate	0.2347	[0.1608-1.3682]	2.5596	[0.1608-5.0613]	**0.0083**	**0.0470**
xylose	3.2865	[0.5342-15.8302]	0.4246	[0.0779-1.4695]	**0.0000**	**0.0016**